SOP-103: DART Editing for Bacterial Genome Engineering via Conjugation

Organism: __

Version: 1.0
Effective Date: __
Author:
Approved by: __

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1. PURPOSE AND SCOPE

1.1 Purpose

Project-specific purpose:

1.2 Process Overview

This SOP describes RNA-guided DNA insertion using CRISPR-Associated Transposase (CAST) systems delivered via bacterial conjugation for genome engineering of non-model gut microbiome bacteria.

CAST-Mediated Integration Workflow:

PREREQUISITE: Vector Construction - gRNA design and cloning completed via Gibson Assembly SOP - DART vector verified and transformed into donor strain - Donor culture prepared and ready for conjugation - **Note:** This SOP begins with a verified DART plasmid in donor cells

STEP 1: Conjugation-Based Delivery - Prepare recipient bacterial culture from gut microbiome - Perform bacterial mating between donor and recipient cells - Plate on selective medium (no DAP, with integration marker) - Incubate at 30°C for 24-48 hours - **Purpose:** Transfer CAST machinery to target bacteria

STEP 2: Selection and Clonal Isolation - Select transconjugants on antibiotic plates - Screen colonies by external-external PCR - Re-streak positive colonies to ensure clonality - Isolate clonal integrants with only integration allele - **Purpose:** Obtain pure clones with genomic insertions

STEP 3: Integration Verification - PCR amplification of genome-transposon junctions - Sanger sequencing to confirm integration site and orientation - Verify 5-bp target site duplication (TSD) - Confirm absence of wild-type allele - **Purpose:** Validate on-target integration events

Key Advantages of CAST Systems: - RNA-programmable targeting (no fixed recognition sequences) - High integration efficiency (50-99% for 1-2 kb payloads) - No requirement for homologous recombination machinery - Suicide vector eliminates need for plasmid curing - Compatible with diverse Gram-negative bacteria - Stable, single-copy chromosomal integration

Mechanism: CAST systems use a TniQ-Cascade complex to bind 32-bp target sequences via crRNA guide. The TnsABC transposase catalyzes non-replicative cut-and-paste insertion of the mini-transposon ~49 bp downstream of the target site. Integration generates 5-bp target site duplications (TSDs) and predominantly T-RL orientation products (>90%).

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2. PROJECT INFORMATION

Project Name: __

Target Bacterium/Bacteria: - Source: _ - *Species/Strain (if known):* _ - Gram stain: ☐ Negative ☐ Positive ☐ Unknown

Genomic Integration Site: _ - *Target gene/locus: - Genomic coordinates:* _ **to** - Integration purpose: ☐ Gene knockout ☐ Reporter insertion ☐ Promoter capture

DART Vector System: _ - *Backbone: - CAST system: - Plasmid selection: - Integration selection:* _

Payload Information: - Size: __ **bp - Components: __** - Expected orientation: ☐ T-RL (preferred) ☐ T-LR ☐ Either

Donor Strain: _ - *Strain: - Genotype:* _ - DAP requirement: ☐ Yes ☐ No

Recipient Strain(s): - Strain ID: _ - *Isolation source: - Growth temperature: 30°C - Growth medium:* _

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3. MATERIALS AND EQUIPMENT

3.1 Reagents and Media

Category	Item	Specification / Notes
Plasmids & Strains	☐ DART plasmid construct	Vector with custom gRNA and payload
	☐ Donor strain competent cells	DAP-dependent (e.g., WM3064, MFDpir+)
	☐ Recipient bacterial culture	Target bacteria for genome editing
Antibiotics (1000× stocks)	☐ Spectinomycin (100 mg/mL)	For donor selection during cloning
	☐ Selection antibiotic	As appropriate for transconjugant selection
	☐ Diaminopimelic acid (DAP, 50 mg/mL)	For donor strain growth (required)
Growth Media	☐ LB broth	Standard lysogeny broth
	☐ LB agar plates	For conjugation and selection
	☐ LB + DAP (0.3 mM final)	For donor strain maintenance
	☐ LB + appropriate antibiotics	For selection as specified
	☐ SOC or LB (recovery medium)	For post-transformation recovery
	☐ Recipient-specific growth medium	As required for target organism
DNA Purification Kits	☐ Plasmid miniprep kit	For plasmid extraction
	☐ PCR purification kit	For PCR product cleanup
	☐ Genomic DNA extraction kit	For gDNA extraction
General Lab Supplies	☐ PBS (1×, pH 7.4)	Gibco #10010023 or make fresh
	☐ Sterile 0.22 µm filters	For media sterilization
	☐ Sterile spreader or plating beads	For plating bacteria
	☐ Pipette tips (sterile)	P2, P10, P20, P200, P1000
	☐ Microcentrifuge tubes (1.7 mL)	For reactions and storage
	☐ 50 mL conical tubes	For cultures
	☐ Petri dishes (100 × 15 mm)	Sterile, for plating
	☐ Cryovials	For glycerol stocks
	☐ Sterile inoculation loops	Single-use plastic loops

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4. SAFETY AND PRECAUTIONS

- [ ] Wear appropriate PPE (lab coat, gloves, safety glasses)
- [ ] Work in biosafety cabinet when handling bacterial cultures
- [ ] Mealworm gut microbiome may contain BSL-2 organisms—follow institutional biosafety protocols
- [ ] UV protection when using gel transilluminator
- [ ] Gentamycin is toxic—handle with care, avoid inhalation or skin contact

- [ ] Autoclave all biological waste before disposal
- [ ] Follow institutional guidelines for GMO handling and disposal
- [ ] DAP-dependent donor strain cannot survive outside laboratory—contains biocontainment feature

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5. PART 1: CONJUGATION-BASED DELIVERY

7.1 Overview

Transfer pBFC0619-gRNA from E. coli WM3064 donor to recipient gut bacteria via bacterial conjugation. Select for transconjugants on gentamycin (no DAP), preventing donor growth.

Mating Method: ☐ Filter mating ☐ Plate mating (direct mix)

7.2 Prepare Recipient Bacterial Culture

Date: __

Recipient Strain: __

1. Inoculate fresh recipient culture from frozen stock or plate
2. Grow in __ **mL** medium
3. Temperature: 30°C
4. Time: __ hours (to mid-log phase, OD600 = 0.4-0.8)
5. Measure OD600: __

Growth Conditions: - Medium: _ - *Incubation time:* _ **hours - Starting OD600:** - Final OD600: __ (mid-log preferred)

✓ **QC Checkpoint:** Recipient culture in exponential growth phase (OD600 = 0.4-0.8)

5.3 Perform Bacterial Conjugation (Plate Mating Method)

Date: __

Mating Reaction:

1. **Prepare donor cells:**
    - Harvest 1 mL donor culture
    - Pellet at 5,000g for 3 minutes
    - Wash with 1 mL sterile PBS (pH 7.4)
    - Pellet again, resuspend in 100 µL PBS
    - Measure OD600: __
2. **Prepare recipient cells:**
    - Harvest 1 mL recipient culture (from Step 5.2)
    - Pellet at 5,000g for 3 minutes
    - Wash with 1 mL sterile PBS
    - Pellet again, resuspend in 100 µL PBS
    - Measure OD600: __
3. **Prepare mating mixtures:**

    Mix donor and recipient (Conjugation):
    - Combine donor and recipient in 1:1 ratio (by OD or volume)
    - Example: 50 µL donor + 50 µL recipient
    - Mix gently by pipetting

Donor only (Control):

- 50 µL donor cells in PBS

Recipient only (Control):

- 50 µL recipient cells in PBS

4. **Spot on conjugation plate:**
   - Prepare LB agar plate (no antibiotics, with DAP if donor requires)
   - Spot each mixture separately on the same plate (label positions):
     - **Conjugation mix:** 10-50 µL in position 1
     - **Donor only:** 10-50 µL in position 2
     - **Recipient only:** 10-50 µL in position 3
   - Allow all spots to dry completely in biosafety cabinet (10-15 min)
5. **Incubate for mating:**
   - Incubate plate at 30°C for 6-24 hours
   - Optimal time: __ hours (typically 18-24 h)

Conjugation Conditions: - Donor:Recipient ratio: __ **:** - Mating time: __ hours - Temperature: 30°C

✓ **QC Checkpoint:** All three mating spots visible on plate after incubation

5.4 Select for Transconjugants

Date: __

1. **Harvest mating spots:**
   - **Conjugation spot:** Add 500 µL sterile PBS, resuspend by pipetting, transfer to tube labeled "Conjugation"
   - **Donor-only spot:** Add 500 µL sterile PBS, resuspend by pipetting, transfer to tube labeled "Donor only"
   - **Recipient-only spot:** Add 500 µL sterile PBS, resuspend by pipetting, transfer to tube labeled "Recipient only"
2. **Prepare serial dilutions for each sample:**
   - 10^{0}: Use 100 µL undiluted
   - 10^{-1}: 100 µL sample + 900 µL PBS
   - 10^{-2}: 100 µL from 10^{-1} + 900 µL PBS
   - 10^{-3}: 100 µL from 10^{-2} + 900 µL PBS
3. **Plate on selective medium:**

   For **each sample** (Conjugation, Donor-only, Recipient-only), plate 100 µL of each dilution on:
   - **Selection plates:** LB + __ (antibiotic), NO DAP ← transconjugants only
   - **Total count plates (optional):** LB only (no antibiotic) ← total viable cells

   Spread evenly with sterile beads or spreader. Allow to dry completely.
4. **Expected results:**
   - **Conjugation plates (LB + antibiotic, no DAP):** Transconjugant colonies
   - **Donor-only plates (LB + antibiotic, no DAP):** NO growth (donor requires DAP)
   - **Recipient-only plates (LB + antibiotic, no DAP):** NO growth (no resistance marker)
5. **Incubate:**
   - Temperature: 30°C
   - Time: 24-48 hours (until colonies visible)

Selection Conditions: - Antibiotic: __ **at** µg/mL - DAP included: ☐ Yes ☐ No - Incubation temperature: 30°C - Incubation time: __ hours

Colony Counts:

Sample	Plate Type	Dilution	Colonies Counted	CFU/mL	Notes
Conjugation	LB + antibiotic (no DAP)	10^{0}	__	__	Transconjugants
Conjugation	LB + antibiotic (no DAP)	10^{-1}	__	__	Transconjugants
Conjugation	LB + antibiotic (no DAP)	10^{-2}	__	__	Transconjugants
Conjugation	LB only	10^{0}	__	__	Total cells
Donor only	LB + antibiotic (no DAP)	All	__	__	Should be 0
Recipient only	LB + antibiotic (no DAP)	All	__	__	Should be 0

Conjugation Efficiency: __ % (transconjugants / total cells × 100)

✓ **QC Checkpoint:** Transconjugant colonies visible on conjugation plates, NO growth on donor-only and recipient-only control plates

Note: If donor-only plates show growth, DAP may have contaminated the medium. If recipient-only plates show growth, the recipient may have native antibiotic resistance—test a higher antibiotic concentration.

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6. PART 2: COLONY SELECTION AND CLONAL ISOLATION

6.1 Overview

Screen transconjugants by colony PCR to identify clones with successful genomic integration. Re-streak to ensure clonality, as initial colonies may be heterogeneous.

Expected Integration Products: - T-RL orientation (preferred, >90%): Transposon right end proximal to target - T-LR orientation (rare, <10%): Transposon left end proximal to target - 5-bp target site duplication (TSD) flanking insertion

6.2 Screen Colonies by PCR

Date: __

Screening Strategy: External-external PCR (genome primers flanking insertion site)

1. **Pick colonies:**
    - Select 10-20 well-isolated colonies from LB + antibiotic plates
    - Resuspend each in 40 µL sterile water in PCR strip tubes
    - Label tubes: Colony #1, #2, #3, etc.
2. **Archive colonies:**
    - Using fresh LB + antibiotic plate, spot 1 µL from each resuspension
    - Allow to dry, then incubate at 30°C overnight as backup
3. **Prepare cell lysate:**
    - Heat resuspensions at 95°C for 10 minutes
    - Cool to room temperature
    - Dilute 1:20 in water (2 µL lysate + 38 µL water)

Colony Lysates:

Colony #	Lysate Prepared?	Spotted on Backup Plate?
1-5	☐	☐
6-10	☐	☐
11-15	☐	☐
16-20	☐	☐

6.3 External-External PCR for Integration Detection

Date: __

Primer Design: - **Upstream*Fwd:*** *Binds genome ~500 bp upstream of target site* - ***Downstream*Rev:** Binds genome ~500 bp downstream of integration site - Expected WT product: ~__ **bp (no insertion) - Expected integration product: ~** bp (includes payload + flanking sequence)

Primers:

Primer Name	Sequence (5' → 3')	Tm	Location
Upstream_Fwd	_ _	_°C	Genome, upstream
Downstream_Rev	_ _	_°C	Genome, downstream

PCR Reaction (25 µL per colony):

Component	Volume per Reaction	Mastermix for 22 Reactions
Diluted lysate	2 µL	N/A
Upstream_Fwd (10 µM)	1.25 µL	27.5 µL
Downstream_Rev (10 µM)	1.25 µL	27.5 µL
Q5 Reaction Buffer (5×)	5 µL	110 µL
dNTPs (10 mM)	0.5 µL	11 µL
Q5 DNA Polymerase	0.25 µL	5.5 µL
Nuclease-free water	14.75 µL	324.5 µL

Prepare mastermix for all reactions plus 2 extra (20 colonies + WT control + no-template control)

PCR Cycling Conditions:

```
1. 98°C - 30 sec (initial denaturation)
2. 30 cycles:
   - 98°C - 10 sec
   - ____°C - 20 sec (annealing, adjust based on primer Tm)
   - 72°C - 120 sec (extension)
3. 72°C - 2 min (final extension)
4. 4°C - hold
```

Controls: - **WT control:** Genomic DNA from recipient strain (no integration) - Expected band: ~_ _ bp (no insertion) - **No-template control:** Water instead of lysate - Expected: No band

6.4 Gel Electrophoresis and Analysis

Date: __

1. Add 5 µL of 6× loading dye to each PCR reaction

2. Load 10 µL per well on 1% agarose gel (0.8% for larger products)
3. Include 1 kb DNA ladder in first and last lanes
4. Run at 120 V for 40-50 minutes (until bands well-separated)
5. Image gel using UV or blue-light transilluminator

Expected Results: - **Integration positive:** Single band at ~__ bp (includes payload) - **Wild-type:** Band at ~__ bp (no insertion) - **Heterogeneous:** Both WT and integration bands present - **No integration:** Only WT band

Screening Results:

Colony #	WT Band?	Integration Band?	Interpretation	Select for Re-streak?
1	☐ Yes ☐ No	☐ Yes ☐ No	☐ Integration ☐ WT ☐ Mixed	☐ Yes ☐ No
2	☐ Yes ☐ No	☐ Yes ☐ No	☐ Integration ☐ WT ☐ Mixed	☐ Yes ☐ No
3	☐ Yes ☐ No	☐ Yes ☐ No	☐ Integration ☐ WT ☐ Mixed	☐ Yes ☐ No
4	☐ Yes ☐ No	☐ Yes ☐ No	☐ Integration ☐ WT ☐ Mixed	☐ Yes ☐ No
5	☐ Yes ☐ No	☐ Yes ☐ No	☐ Integration ☐ WT ☐ Mixed	☐ Yes ☐ No
6-20	...	...	...	...

✓ **QC Checkpoint:** At least 3-5 colonies show integration band

6.5 Re-streak for Clonal Purity

Date: __

Rationale: Initial colonies are often heterogeneous (contain both integrated and WT alleles). Re-streaking ensures clonal populations.

1. Select 3-5 colonies showing integration bands (from Step 6.4)
2. From backup plates, pick well-isolated colonies
3. Streak for single colonies on fresh LB + antibiotic plates
4. Incubate at 30°C for 24-48 hours

Re-streaked Clones:

Original Colony #	Re-streak Plate ID	Incubation Time
Colony #__	Plate A	__ hours
Colony #__	Plate B	__ hours
Colony #__	Plate C	__ hours

1. After growth, pick 3-5 single colonies from each re-streak plate
2. Repeat colony PCR screening (Steps 6.2-6.4)
3. Identify clones with ONLY integration band (no WT band)

Second-Round Screening Results:

Re-streak Plate	Colony #	WT Band?	Integration Band?	Clonal?
Plate A	1	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Plate A	2	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Plate B	1	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Plate B	2	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Plate C	1	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No

✓ **QC Checkpoint:** At least one clonal integrant identified (integration band only, no WT)

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7. PART 3: INTEGRATION VERIFICATION

7.1 Overview

Confirm on-target integration by sequencing genome-transposon junctions. Verify integration orientation (T-RL vs T-LR) and precise insertion site.

7.2 Junction PCR for Sequencing

Date: __

Strategy: Amplify genome-transposon junctions using genome-specific and transposon-specific primers

Primer Pairs:

5' Junction (Upstream): - Genome*Fwd: Binds genome ~300 bp upstream of target - TnsR*Rev: Binds transposon right end

3' Junction (Downstream): - TnsL*Fwd: Binds transposon left end*
- *Genome*Rev: Binds genome ~300 bp downstream

Primers:

Primer Name	Sequence (5' → 3')	Tm	Target
Genome_Fwd	_ _	_ °C	Genome
TnsR_Rev	5'-CGCCAGGGTTTTCCCAGTC-3'	60°C	Transposon R end
TnsL_Fwd	5'-CTGTAGCGCGTTTTCATCGG-3'	60°C	Transposon L end
Genome_Rev	_ _	_ °C	Genome

Select 2-3 clonal integrants from Step 6.5

1. Inoculate clones in 5 mL __ medium + antibiotic
2. Grow overnight at 30°C
3. Extract genomic DNA using Wizard Genomic DNA Purification Kit
4. Quantify DNA

gDNA Yields:

Clone ID	Concentration (ng/µL)	A260/A280	Pass/Fail
Clone #__	__	__	☐ Pass ☐ Fail
Clone #__	__	__	☐ Pass ☐ Fail
Clone #__	__	__	☐ Pass ☐ Fail

PCR Reaction for Each Junction (25 µL):

Component	Volume
gDNA (10-50 ng)	2 µL
Forward primer (10 µM)	1.25 µL
Reverse primer (10 µM)	1.25 µL
Q5 Reaction Buffer (5×)	5 µL
dNTPs (10 mM)	0.5 µL
Q5 DNA Polymerase	0.25 µL
Nuclease-free water	14.75 µL

PCR Cycling Conditions:

```
1. 98°C - 30 sec
2. 30 cycles:
   - 98°C - 10 sec
   - ____°C - 20 sec (annealing)
   - 72°C - 30 sec (extension: ~300-500 bp product)
3. 72°C - 2 min
4. 4°C - hold
```

Run on 1.5% agarose gel - Expected 5' junction product: ~__ bp - Expected 3' junction product: ~__ bp

7.3 Gel Extract and Sequence Junctions

Date: __

1. Excise junction PCR products from gel
2. Gel extract using QIAquick Gel Extraction Kit
3. Elute in 30 µL elution buffer
4. Quantify DNA

Purified Junction Products:

Clone ID	Junction	Concentration (ng/µL)	Pass/Fail
Clone #__	5'	__	☐ Pass ☐ Fail
Clone #__	3'	__	☐ Pass ☐ Fail

1. Submit for Sanger sequencing
    - Use PCR primers (Genome*Fwd, Genome*Rev) for sequencing
    - Sequence both junctions for each clone

7.4 Analyze Sequencing Results

Date: __

Expected Features: - **5-bp Target Site Duplication (TSD):** Genomic sequence duplicated on both sides of insertion - **Integration distance:** ~49 bp downstream of target site (3' end of 32-bp target) - **Orientation:** T-RL (right end proximal) or T-LR (left end proximal) - **Intact transposon ends:** Proper junction between genome and transposon

Sequencing Analysis:

Clone ID	5' Junction Correct?	3' Junction Correct?	TSD Identified?	Distance from Target	Orientation	Pass/Fail
Clone #__	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	_ bp	☐ T-RL ☐ T-LR	☐ PASS ☐ FAIL
Clone #__	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	_ bp	☐ T-RL ☐ T-LR	☐ PASS ☐ FAIL
Clone #__	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	_ bp	☐ T-RL ☐ T-LR	☐ PASS ☐ FAIL

5-bp TSD Sequence: 5'-__-3'

Validated clone(s): Clone #_, *Clone #, Clone #_*

Selected for archiving: Clone #__

✓ **Final QC Checkpoint:** At least one fully validated clone with correct junctions and on-target integration

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8. STRAIN ARCHIVING

Date: __

Validated Clone ID: Clone #__

Strain Name: __

Genotype: - Parent strain: _ - *Modification: - Integration site: - Payload:* _ - Orientation: ☐ T-RL ☐ T-LR

Description:

Glycerol Stock Preparation:

1. Inoculate validated clone in 5 mL __ medium + antibiotic
2. Grow overnight at 30°C to saturation (OD600 >2)
3. Mix 500 µL culture + 500 µL sterile 50% glycerol in cryovial
4. Vortex thoroughly to mix
5. Label cryovials clearly
6. Flash freeze in liquid nitrogen or dry ice/ethanol bath
7. Store at -80°C

Storage Locations:

Vial	Box	Position	Freezer	Date
1	__	__	__	__
2	__	__	__	__
3	__	__	__	__

Backup Location: __

Lab Notebook Reference: __

Electronic Files: - Sequencing results: _ - *Gel images: - Plasmid maps:* _

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9. TROUBLESHOOTING

Problem	Possible Cause	Solution
No colonies after gRNA cloning	• Incomplete digestion • Ligation failed • Non-competent cells	• Verify complete digestion by gel • Check ligase activity with control • Use fresh competent cells
High background on no-insert control	• Vector self-ligation • Incomplete digestion	• Increase digestion time to 3-4 hours • Add alkaline phosphatase (CIP) to digest • Use higher insert:vector ratio
Incorrect gRNA sequence	• Wrong oligos ordered • Cloning error	• Re-design and re-order oligos • Sequence more clones • Check for mutations in synthesis
Donor strain grows on -DAP plates	• DAP contamination • Revertant strain	• Prepare fresh media and plates • Verify donor strain genotype • Use fresh donor stock
No transconjugants after conjugation	• Donor/recipient not viable • Insufficient mating time • Wrong antibiotic concentration • Recipient naturally resistant	• Verify donor and recipient OD600 • Extend mating time to 24 hours • Optimize antibiotic concentration (MIC) • Test recipient on antibiotic plates
Donor grows on selection plates (no DAP)	• DAP contamination in medium • Integration into donor genome	• Remake selection plates without DAP • Verify plates are DAP-free • Use fresh donor culture
Low conjugation efficiency (<0.1%)	• Poor mating conditions • Wrong donor:recipient ratio • Incompatible strains	• Optimize mating time (6-24 h) • Try different ratios (1:1, 1:10, 10:1) • Increase mating temperature slightly • Use filter mating method instead
All colonies are wild-type (no integration)	• gRNA not functional • Transposase not expressed • Target site not accessible	• Re-design gRNA to different target • Verify payload integrity by sequencing • Test different genomic sites • Extend incubation time at 30°C
Integration efficiency very low (<10%)	• Suboptimal temperature • Large payload • Target site chromatin structure	• Incubate at 25-30°C instead of 37°C • Screen more colonies (30-50) • Try alternative target sites • Extend incubation to 48 hours
Heterogeneous colonies (mixed WT + integration)	• Insufficient growth time • Clones not pure	• Re-streak colonies 2-3 times • Pick smaller, younger colonies • Extend incubation between re-streaks
PCR gives no product	• Primers don't bind • Poor DNA template quality • Wrong annealing temperature	• Verify primer sequences • Re-extract DNA or use fresh lysate • Optimize annealing temperature • Use touchdown PCR
Wrong integration site (off-target)		

Problem	Possible Cause	Solution
	• Off-target sites in genome • Non-specific gRNA	• Re-design gRNA with BLAST check • Avoid targets with >2 off-targets • Sequence more clones to find on-target
Both T-RL and T-LR orientations detected	• Normal behavior for CASTs • T-RL is preferred (>90%)	• Screen more colonies to find T-RL • Both orientations are functional • Select preferred orientation if needed
No 5-bp TSD detected in sequence	• Sequencing quality issue • Not a CAST integration event	• Re-sequence with higher quality • Verify integration by junction PCR • Check for contamination
Integration product includes vector backbone	• Cointegrate formation • Incomplete excision	• This is rare for Type I-F CASTs • Verify by long-read sequencing • Use alternative clone
Antibiotic selection doesn't work	• Recipient has native resistance • Antibiotic degraded	• Determine MIC for recipient strain • Use higher concentration • Make fresh antibiotic stocks • Try alternative selection marker

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10. TIMELINE

Estimated timeline for complete protocol:

Week	Activities	Checkpoints
Week 1	Prepare donor strain with DART plasmid	Verified donor culture ready
Week 2	Grow recipient bacteria, perform conjugation	Transconjugant colonies
Week 3	Screen by PCR, re-streak for clonality	Clonal integrants identified
Week 4	Junction PCR, sequencing, validation	Validated strain archived

Total time: ~4 weeks (from donor preparation to validated strain)

Hands-on time: ~6-8 days total

Note: Timeline assumes: - DART plasmid already constructed and verified (Gibson Assembly SOP completed) - Sanger sequencing turnaround: 1-2 days - Recipient bacteria can be cultured to mid-log in <24 hours

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11. REFERENCES

1. Gelsinger, D. R. et al. (2024). Bacterial genome engineering using CRISPR-associated transposases. *Nature Protocols*, 19(3), 752-790. https://doi.org/10.1038/s41596-023-00927-3
2. Klompe, S. E., Vo, P. L. H., Halpin-Healy, T. S. & Sternberg, S. H. (2019). Transposon-encoded CRISPR–Cas systems direct RNA-guided DNA integration. *Nature*, 571(7764), 219–225.
3. Strecker, J. et al. (2019). RNA-guided DNA insertion with CRISPR-associated transposases. *Science*, 365(6448), 48–53.

4. Additional references:
    - __
    - __
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12. REVISION HISTORY

Version	Date	Author	Changes
1.0	__	__	Initial version

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13. APPENDICES

Appendix A: Primer Design Worksheet

Project: __

Target Genomic Locus: __

Note: gRNA design and cloning is covered in the Gibson Assembly SOP. This worksheet covers only PCR verification primers.

A.1 PCR Screening Primers

External-External Primers (for clonal screening):

Primer Name	Sequence (5' → 3')	Length	Tm	Location
Upstream_Fwd		__ bp	_°C	~500 bp upstream of target
Downstream_Rev		__ bp	_°C	~500 bp downstream of integration

Expected Products: - Wild-type (no integration): ~__ bp - Integration: ~__ bp (includes payload)

A.2 Junction Sequencing Primers

Genome-Transposon Junction Primers:

Primer Name	Sequence (5' → 3')	Length	Tm	Target
Genome_Fwd		__ bp	_°C	Genome, ~300 bp upstream
TnsR_Rev	5'-CGCCAGGGTTTTCCCAGTC-3'	19 bp	60°C	Transposon R end (constant)
TnsL_Fwd	5'-CTGTAGCGCGTTTTCATCGG-3'	20 bp	60°C	Transposon L end (constant)
Genome_Rev		__ bp	_°C	Genome, ~300 bp downstream

Expected Products: - 5' junction (Genome*Fwd* + *TnsR*Rev): ~__ bp - 3' junction (TnsL*Fwd* + *Genome*Rev): ~__ bp

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A.4 Sequencing Primers

For Plasmid Verification:

Primer Name	Sequence (5' → 3')	Use
CAST*array*seq	5'-GAAACCGTTCGTCATCATCGTC-3'	Verify gRNA spacer in CRISPR array

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Appendix B: Reagent Preparation Recipes

All solutions should be filter-sterilized (0.22 µm) or autoclaved as indicated.

Reagent	Recipe	Storage
50% Glycerol (sterile)	Mix 250 mL glycerol + 250 mL MQ water. Autoclave.	4°C, <6 months
10% Glycerol (sterile)	Mix 50 mL glycerol + 450 mL MQ water. Autoclave or filter-sterilize.	4°C, <6 months
1× PBS (pH 7.4)	8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per 1 L water. Autoclave.	RT, 12 months
Spectinomycin (100 mg/mL)	Dissolve 5 g powder in 50 mL water. Filter-sterilize (0.22 µm). Aliquot 1 mL.	-20°C, <12 months
Gentamycin (10 mg/mL)	Dissolve 0.5 g powder in 50 mL water. Filter-sterilize. Aliquot 1 mL.	-20°C, <12 months
DAP (50 mg/mL)	Dissolve 2.5 g diaminopimelic acid in 50 mL water. Filter-sterilize. Aliquot 1 mL.	-20°C, <12 months
TAE Buffer (50×)	242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0). Adjust to 1 L.	RT, 12 months
1% Agarose Gel	Dissolve 1 g agarose in 100 mL 1× TAE. Microwave until clear. Cool to 60°C, add 10 µL SYBR Safe. Pour.	Use fresh
LB Medium	10 g tryptone, 5 g yeast extract, 10 g NaCl per 1 L water. Autoclave.	RT, 1 month
LB Agar	LB medium + 15 g agar per 1 L. Autoclave. Cool to 55°C, add antibiotics. Pour plates.	4°C, 1 month

Working Antibiotic Concentrations:

Antibiotic	Stock	Working Concentration	Use
Spectinomycin	100 mg/mL	100 µg/mL	Donor selection (for plasmid maintenance)
Selection antibiotic	As appropriate	Determine MIC for recipient	Transconjugant selection
DAP	50 mg/mL	0.3 mM (300 µM)	Donor growth supplement

Note: Determine the minimum inhibitory concentration (MIC) of the selection antibiotic for your recipient strain before starting conjugation experiments.

Appendix C: Data Recording Templates

Template 1: Conjugation Record

Date: __
Donor strain:
Recipient strain:
Donor:Recipient ratio: : __
Mating time: hours at 30°C
Selection: LB + __ **(__** µg/mL), no DAP

Colony counts: - Transconjugants (LB + Gent): __ **CFU/mL - Total cells (LB only):** CFU/mL - Conjugation efficiency: __ %

Result: ☐ Success ☐ Failed
Notes: _

Template 2: PCR Screening Record

Date: __
Primer set: **Upstream*Fwd* + *Downstream*Rev**
Template: **Colony lysates from transconjugants**
Polymerase: **Q5 or OneTaq**
Annealing temp: **°C**
Extension time: **__** sec
Cycles: 30

Gel Results: - Expected WT band: _ *bp - Expected integration band: bp - Colonies screened: - Integration-positive colonies: _*

Result: ☐ Success ☐ Optimize
Notes: _

Template 3: Sequencing Verification Record

Date: __
Clone ID: **Clone #**
Junction: ☐ **5' (upstream)** ☐ **3' (downstream)**
Primer used:
Sequencing service: **__**

Results: - 5-bp TSD identified: ☐ Yes ☐ No → Sequence: 5'-_***-3' - Distance from target:*** ___ bp (expected: ~49 bp) - Orientation: ☐ T-RL ☐ T-LR - Junction sequence correct: ☐ Yes ☐ No

Overall: ☐ PASS ☐ FAIL
Notes: _

Template 4: Glycerol Stock Record

Date: __
Strain name:
Clone ID: **Clone #**
Parent strain:
Modification: **at __**

Storage: - Vial 1: Box _, *Position , Freezer - Vial 2: Box , Position , Freezer - Vial 3: Box , Position , Freezer _*

Lab notebook page: _
Electronic files: _

Appendix D: Quick Reference - CAST Integration Mechanism

CAST (CRISPR-Associated Transposase) Integration Features:

1. **Target Recognition:**
   - gRNA: 32-bp target sequence
   - PAM: 5'-CN-3' (C = C/G/T/A, N = any) for most Type I-F CAST systems
   - TniQ-Cascade complex binds target
2. **Integration Site:**
   - Distance: ~49 bp downstream of target (3' end)
   - Variability: ±5 bp depending on local sequence
   - Target site is NOT disrupted
3. **Integration Products:**
   - **T-RL orientation (preferred, >90%):** Right end proximal to target
   - **T-LR orientation (rare, <10%):** Left end proximal to target
   - **5-bp Target Site Duplication (TSD):** Flanks insertion on both sides
4. **Transposon Structure:**
   - Mini-transposon: Payload flanked by L and R ends
   - Payload size: Optimal 0.5-2 kb, functional up to >10 kb
   - Transposon ends: ~200 bp (L) and ~60 bp (R, can be truncated to 57 bp)
5. **Efficiency:**
   - Single-target integration: 50-99% (depending on conditions)
   - Temperature effect: Lower temp (25-30°C) improves large payloads
   - Specificity: >99% on-target for well-designed gRNAs
6. **Selection:**
   - Suicide vector: No plasmid curing needed
   - Integration selection: Payload marker (defined in Project Information)
   - Transconjugants: Selected on antibiotic + no DAP

END OF SOP

Performed by: _
Date:
Signature: _

Reviewed by: _
Date:
Signature: _