| | |
|---|---|
| 01 | **April to June 2026 · Foundation and Setup**<br>Transfection conditions calibrated, seeding density series run, and AREG and SELE selected as trigger targets from the ranking pipeline. |

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|---|---|
| 02 | **July to August 2026 · Construct Build and Validation**<br>Four constructs assembled by Gibson assembly, dual-promoter expression confirmed by FACS, and all four verified by whole-plasmid sequencing. |

| | |
|---|---|
| 03 | **September to October 2026 · Expression and Characterisation**<br>Constructs transfected, RNA purified, cDNA synthesised, and a 93-well RT-qPCR run used to quantify trigger expression and primer specificity. |

# April to June 2026 · Foundation and Setup

**Key Achievements**
Transfection reagent to DNA ratio calibrated to 1:3
Seeding density series run in 96-well format
AREG and SELE selected as trigger targets from the ranking pipeline

## April 20 to 26

- HEK293T stock thawed and expanded; culture conditions and passaging schedule established
- Consumables and plasmid stocks inventoried; 96-well transfection workflow planned

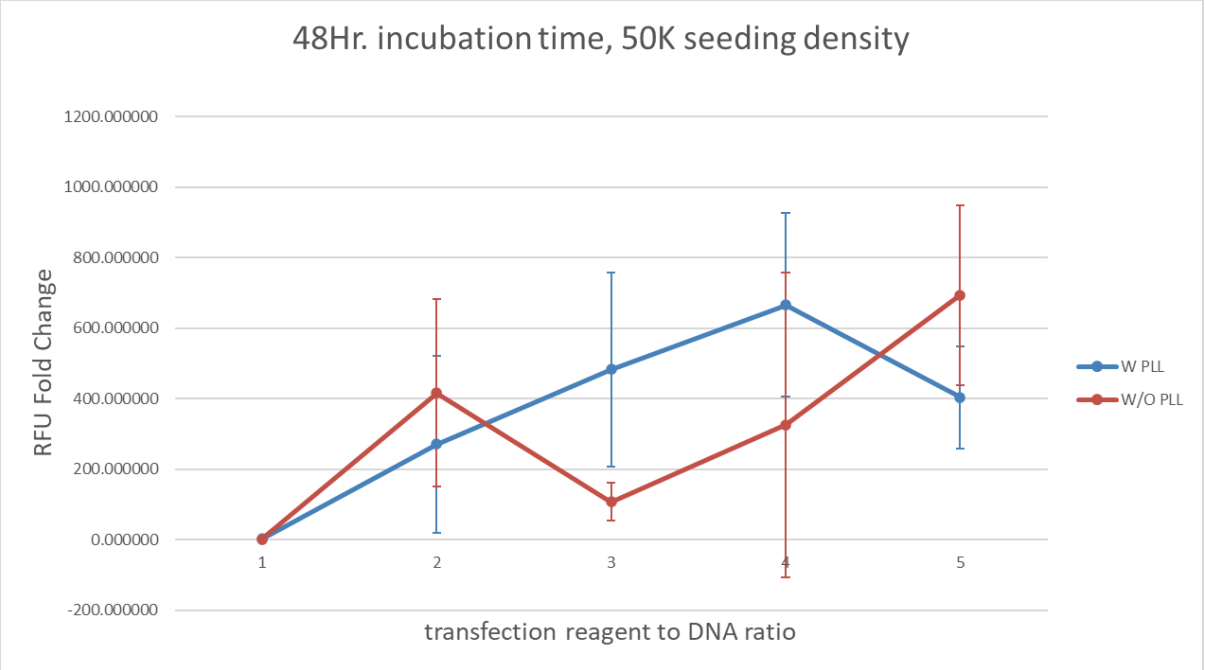
## April 27 to May 3

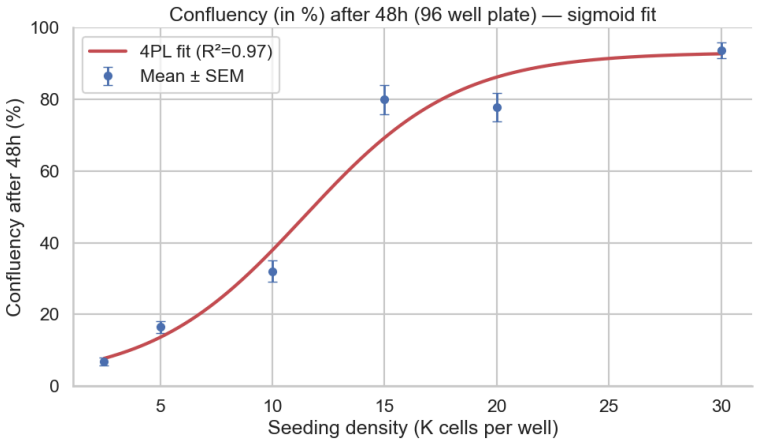
- **Calibration of transfection reagent to DNA ratio (28.04.2026).** HEK293T cells seeded in a 96-well plate and transfected at ratios from 1:1 to 1:5, holding DNA constant
- Poly-lysine (PLL) treated wells compared against untreated wells, on the expectation that better adhesion gives a stronger and more uniform signal
- Fluorescence measured as a proxy for transfection efficiency
- **Result.** The 1:3 ratio gave the best overall performance. Ratios of 1:4 and 1:5 did not improve on it and may have reduced cell health. PLL wells gave the more stable response

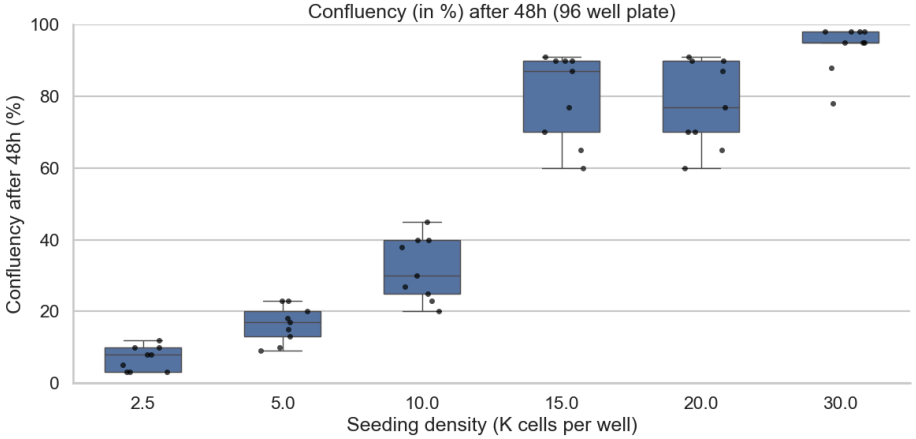
## May 4 to 17

- **Optimisation of HEK293T seeding density.** Cells seeded at 5k, 10k, 15k, 20k and 30k cells per well in a 96-well plate
- Confluency assessed 48 h after seeding, the time point at which transfection readouts are taken

- Imaging collected for each density; quantification pending



### May 18 to June 14

- **Trigger selection.** AREG and SELE chosen as the two ON-gate trigger targets on the basis of their pipeline ranks. AREG scored A+ (single BLAST hit, self only; +4.0 log2FC in ovarian and +2.1 log2FC in peritoneal ectopic lesions). SELE scored B− with consistent upregulation across both lesion subtypes
- The two targets give orthogonal trigger sequences for a two-target proof of concept
- qPCR primer pairs identified for AREG and SELE from published, validated sets; recoded-sequence primers designed in house

### June 15 to July 5

- Construct design finalised: each trigger CDS plus UTRs, with homology arms matching the pDD-HTP4 backbone appended at the 5′ and 3′ ends
- Synonymously recoded CDS variants designed, with wobble-position codons altered throughout the trigger-complementary region, to serve as negative controls
- Because of gene synthesis length constraints, the SELE construct was ordered without 944 nt of its 3′ UTR
- Synthetic fragments ordered from Twist (order 2TYL3F)

## July to August 2026 · Construct Build and Validation

**Key Achievements**
All four constructs assembled, transformed and mini-prepped
Dual-promoter expression confirmed by FACS
Whole-plasmid sequencing passed for three of four constructs; the fourth recovered on resubmission

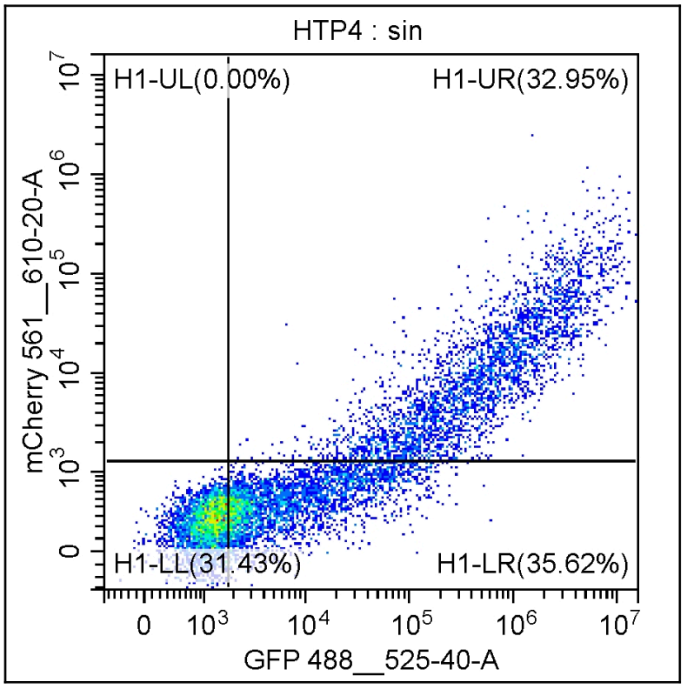
### July 6 to 19

- Twist fragments resuspended per the supplier protocol
- **Gibson assembly.** Inserts cloned into the pDD-HTP4 dual-reporter backbone (EF-1α to biomarker CDS; mCherry/SV40; EGFP/CMV-T7; AmpR, ColE1; 6833 bp), pre-linearised with PmlI. 3:1 insert to vector, 100 fmol total in 10 µL, 45 min at 50 °C; 5 µL of the reaction used for transformation
- Colonies from all four transformations (pDD-HTP4-AREG, -AREG-R, -SELE, -SELE-R) grew on LB+Amp

### July 20 to 26

- **FACS analysis of dual-promoter plasmid expression (21.07.2026).** HEK293T cells transfected with a plasmid carrying both GFP and mCherry cassettes, using

the optimised seeding density and reagent ratio
- Cells analysed by FACS for GFP and mCherry fluorescence, alongside a control transfection and the pDD-HTP3 and pDD-HTP4 backbones
- **Result.** Strong GFP and strong mCherry signal detected. Both promoters are active and the plasmid expresses both reporters, so the construct is suitable for further testing




## July 27 to August 9

- Single colonies picked, cultured and mini-prepped; plasmid DNA recovered from all four constructs
- All four submitted for Plasmidsaurus whole-plasmid sequencing (order 2TYL3F)

## August 10 to 16

- **Sequencing results returned (run 2026-08-11).** AREG carried two mismatches, at position 492 in the pUC origin and position 3123 in the terminator
- AREG-Recoded carried three mismatches, at position 521 in the pUC origin, position 1919 upstream of AREG, and position 3123 in the terminator
- SELE carried a mismatch at position 521 in the pUC origin and the same mismatch in the terminator
- SELE-Recoded failed sequencing: a faint band at roughly 6% of the others, with no assembly, consistent with low DNA input
- None of the mismatches fall in a coding or regulatory element of the insert, so the three passing constructs were carried forward

## August 17 to 30

- pDD-HTP4-SELE-R re-prepped at higher concentration; three colonies (a, b, c) resubmitted as order KKMDJY
- Colony b did not carry the SELE-R gene and was discarded
- Colonies a and c carried the same two mismatches as the other constructs (position 492 in the pUC origin, position 3123 in the terminator); colony a was taken forward

# September to October 2026 · Expression and Characterisation

**Key Achievements**
All four constructs transfected into HEK293T and RNA recovered
cDNA synthesised with a minus-RT control
93-well RT-qPCR run completed; trigger expression and primer specificity quantified

## August 31 to September 13

- **Transfection.** HEK293T cells seeded and transfected with pDD-HTP4-AREG, -AREG-R, -SELE and -SELE-R at the calibrated 1:3 ratio
- Two controls carried in parallel: cells transfected with the empty pDD-HTP4 backbone, and untransfected cells
- Cells harvested 48 h post-transfection for RNA purification

## September 14 to 27

- **mRNA purification** using the Roche High Pure RNA Isolation Kit, including the on-column DNase I digestion (see Protocols)
- RNA eluted in 50 to 100 µl and quantified by NanoDrop; yields ranged from 122 to 932 ng/µL across the six samples
- Eluates kept on ice and stored at −80 °C

## September 28 to October 5

- **cDNA synthesis** from 2 µg total RNA per 20 µl reaction using the qScript cDNA Synthesis Kit, with a minus-RT control prepared from the AREG RNA preparation
- 100 µM primer stocks made and 10 µM working stocks prepared for all twelve oligos
- Plate layout designed: 81 sample wells plus 12 NTC wells, 93 of 96 used
- Six primer-pair master mixes calculated with 10% overage

## October 6 to 12

- **RT-qPCR run.** 93 wells, six primer pairs against eight templates, technical triplicates throughout
- Melt curve run immediately after cycling, as part of the same method
- Data exported and analysed; see Analysis

# Protocols

## Trigger primer sequences

| Primer | Sequence (5′ to 3′) |
|---|---|
| SELE_FW | TCAAGGGCAGTGGACACAGCAA |
| SELE_REV | GGAAACTGCCAGAAGCACTAGG |
| AREG_FW | GCACCTGGAAGCAGTAACATGC |
| AREG_REV | GGCAGCTATGGCTGCTAATGCA |
| SELE_Recoded_FW | GCTTCAGCATACTGCCAACA |
| SELE_Recoded_REV | CCAACCCAAACCCAGACGTTA |
| AREG_Recoded_FW | TCCGGAAAACGAGAGCCTTT |
| AREG_Recoded_REV | ATCGTAATCAGCCCCACTGC |

The SELE and AREG pairs amplify a 70 to 86 bp fragment. Reference gene pairs UBC and TOP1 were run alongside.

## mRNA purification: Roche High Pure RNA Isolation Kit (Cat. No. 11 828 665 001)

**Sample preparation.** Up to 1 × $10^6$ cultured cells were harvested. Adherent HEK293T cells were detached by trypsinisation, pelleted, and resuspended in 200 µl PBS. 400 µl Lysis/Binding Buffer was added and the sample vortexed for 15 s.

1. Lysate pipetted into a High Pure Filter Tube assembly, centrifuged 15 s at 8,000 × g, flowthrough discarded
2. DNase I treatment: 90 µl DNase Incubation Buffer plus 10 µl DNase I per sample; 100 µl added to the filter and incubated 15 min at +15 to +25 °C
3. Wash I: 500 µl Wash Buffer I, 15 s at 8,000 × g
4. Wash II: 500 µl Wash Buffer II, 15 s at 8,000 × g
5. Wash III: 200 µl Wash Buffer II, 2 min at maximum speed (about 13,000 × g) to dry the filter
6. Elution: filter transferred to a clean 1.5 ml tube, 50 to 100 µl Elution Buffer added, 1 min at 8,000 × g
7. Concentration measured by NanoDrop

| Sample | Concentration (ng/µL) |
|---|---|
| AREG | 435 |
| AREG-R | 589 |
| SELE | 885 |
| SELE-R | 566 |
| Empty plasmid | 122 |
| Cells without plasmid | 932 |

## cDNA synthesis: qScript cDNA Synthesis Kit (Quanta BioSciences, Cat. No. 95047)

Each 20 µl reverse-transcription reaction contained 4 µl qScript Reaction Mix (5×), 1 µl qScript Reverse Transcriptase, 2 µg total RNA, and nuclease-free water to volume.

| No. | Sample | Starting conc. (ng/µL) | RNA volume (µL) | $ddH_2O$ (µL) |
|---|---|---|---|---|
| 1 | AREG | 435 | 4.60 | 15.40 |
| 2 | AREG-R | 589 | 3.39 | 16.61 |
| 3 | SELE | 885 | 2.25 | 17.75 |
| 4 | SELE-R | 566 | 3.53 | 16.47 |
| 5 | Empty plasmid | 122 | 15.00 | 0.00 |
| 6 | Cells, no plasmid | 932 | 2.14 | 17.86 |
| 7 | AREG, no RT enzyme | 435 | 4.60 | 15.40 |

Thermal cycler programme: 22 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, hold at 4 °C. cDNA stored at −20 °C.

**Controls.** A minus-RT control was included to assess residual genomic DNA. Because the RNA had already undergone on-column DNase treatment, this control verifies that the qPCR signal reflects reverse-transcribed RNA rather than DNA.

## RT-qPCR: PowerTrack SYBR Green Master Mix

**Run conditions**
Kit: Thermo Fisher PowerTrack SYBR Green Master Mix (A46012)
Reaction volume: 10 µL. Primer supplier: IDT
Instrument: QuantStudio 3, fast mode. ROX is included in the master mix and was set as the passive reference
The kit's Yellow Sample Buffer was added to each template, so every loaded well is visibly green before the run

**cDNA dilution.** The RT reaction contained 2 µg RNA in 20 µl, giving a nominal 100 ng/µL RNA-equivalent stock. This was diluted 1:20 to 5 ng/µL (3 µL cDNA plus 57 µL nuclease-free water), then 1 µL loaded per well for 5 ng RNA-equivalent per reaction.

| Template | Wells | 1:20 cDNA | Yellow Sample Buffer | Working template |
|---|---|---|---|---|
| AREG | 9 | 12 µL | 3 µL | 15 µL |
| AREG-R | 9 | 12 µL | 3 µL | 15 µL |
| SELE | 9 | 12 µL | 3 µL | 15 µL |
| SELE-R | 9 | 12 µL | 3 µL | 15 µL |
| AREG minus RT | 9 | 12 µL | 3 µL | 15 µL |
| Empty plasmid | 18 | 24 µL | 6 µL | 30 µL |
| Cells only | 18 | 24 µL | 6 µL | 30 µL |
| NTC | 12 | 16 µL water | 4 µL | 20 µL |

**Plate design.** 81 sample wells plus 12 NTC wells (6 primer pairs × 2), giving 93 of 96 wells used.

| Sample | Primer pairs run | Wells |
|---|---|---|
| AREG | AREG, UBC, TOP1 | 9 |
| AREG-R | AREG-R, UBC, TOP1 | 9 |

| | | |
|---|---|---|
| SELE | SELE, UBC, TOP1 | 9 |
| SELE-R | SELE-R, UBC, TOP1 | 9 |
| Empty plasmid | all six | 18 |
| Cells, no plasmid | all six | 18 |
| AREG, no RT enzyme | AREG, UBC, TOP1 | 9 |

**Per-well composition.** 5 µL PowerTrack SYBR Green Master Mix (2×), 0.4 µL of each primer at 10 µM for 400 nM final, and 2.95 µL nuclease-free water. That is 8.75 µL of master mix dispensed per well, plus 1.25 µL of dyed template, for 10 µL total.

| Primer pair | Prepare for | 2× mix (µL) | F primer (µL) | R primer (µL) | Water (µL) | Total (µL) | Wells |
|---|---|---|---|---|---|---|---|
| UBC | 26 | 130 | 10.4 | 10.4 | 76.7 | 227.5 | 23 |
| TOP1 | 26 | 130 | 10.4 | 10.4 | 76.7 | 227.5 | 23 |
| AREG | 16 | 80 | 6.4 | 6.4 | 47.2 | 140.0 | 14 |
| AREG-R | 13 | 65 | 5.2 | 5.2 | 38.4 | 113.8 | 11 |
| SELE | 13 | 65 | 5.2 | 5.2 | 38.4 | 113.8 | 11 |
| SELE-R | 13 | 65 | 5.2 | 5.2 | 38.4 | 113.8 | 11 |
| **Total** | **107** | **535** | **42.8** | **42.8** | **315.7** | **936.3** | **93** |

| Stage | Temperature | Time | Cycles |
|---|---|---|---|
| Enzyme activation | 95 °C | 2 min | 1 |
| Denature | 95 °C | 5 s | 40 |
| Anneal and extend | 60 °C | 30 s | 40 |
| Melt curve | 95 to 60 to 95 °C | 15 s · 1 min · 0.075 °C/s | 1 |

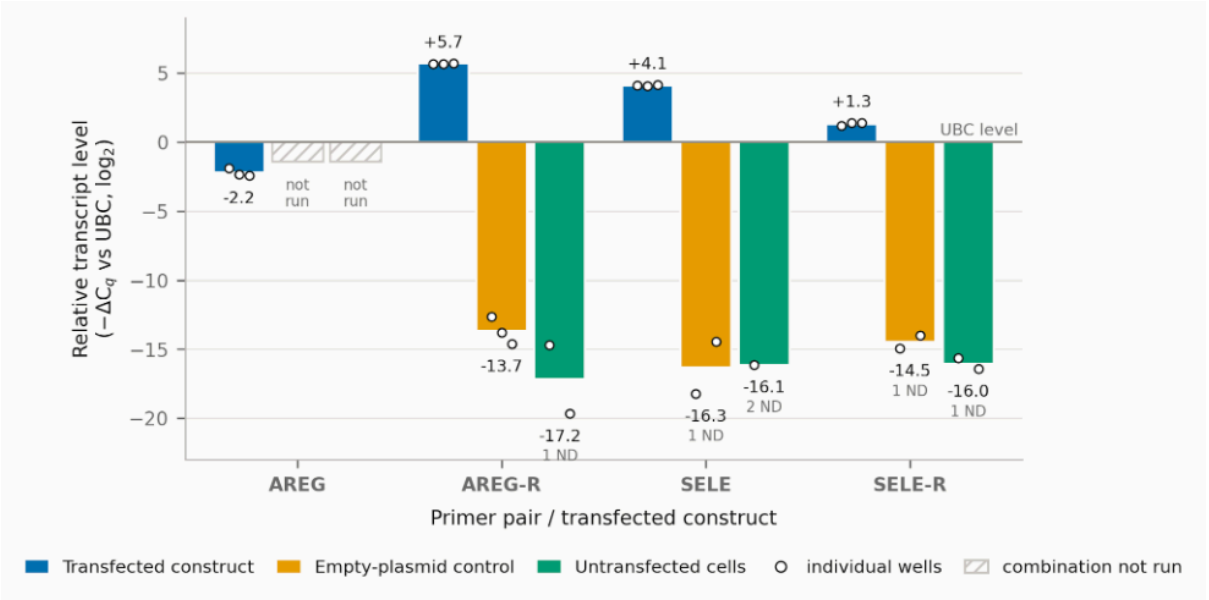
# Analysis

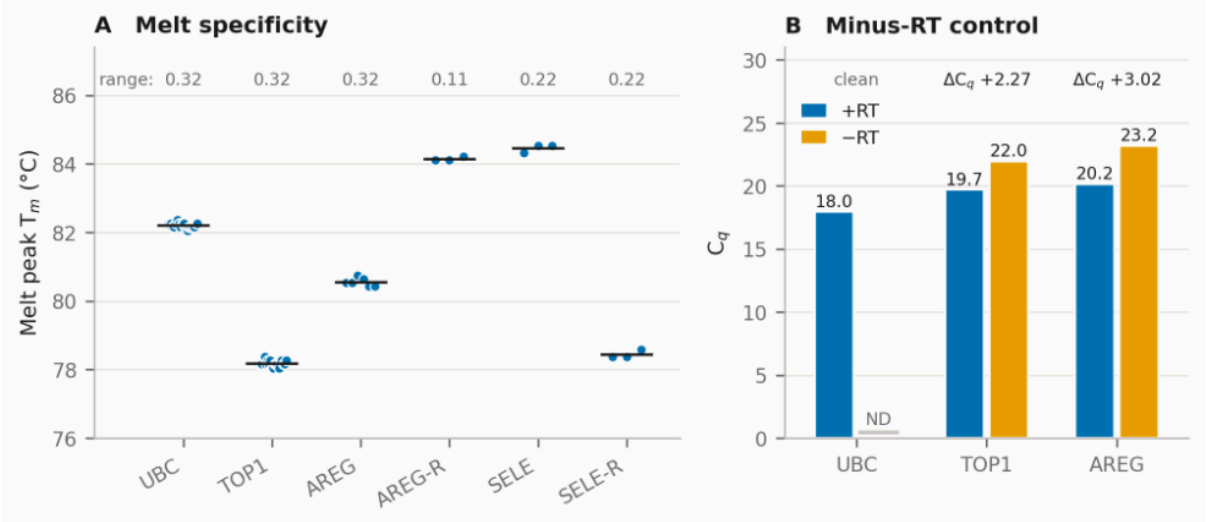
Transcription of the four constructs was quantified by RT-qPCR in transfected HEK293T cells and normalised to the reference gene UBC. TOP1 was assayed as a second reference but excluded: its minus-RT control amplified 2.27 Cq behind the +RT sample, indicating that residual genomic DNA contributed roughly 20% of its signal. UBC's minus-RT control was clean, and UBC varied by only 1.31 Cq across all six templates. AREG-R, SELE and SELE-R were readily detected in their transfected samples (Cq 12.3, 13.2 and 15.5) and effectively absent from both untransfected cells and empty-plasmid controls (Cq 31.8 to 34.7, with one to two of three replicates giving no amplification at all). Normalised to UBC this is a separation of 17.4 to 22.9 Cq, or five to seven orders of magnitude.

| Construct | Cq, transfected | Cq, untransfected | ΔΔCq | Fold over background |
|---|---|---|---|---|
| AREG-R | 12.25 | 34.68 | −22.87 | about $10^7$ |
| SELE | 13.19 | 33.62 | −20.24 | about $10^6$ |
| SELE-R | 15.52 | 33.54 | −17.37 | about $10^5$ |
| AREG | 20.17 | not run | n/a | n/a |

Fold changes are reported to the nearest order of magnitude, because amplification

efficiencies are not yet validated, and as lower bounds, because the control wells sit at the limit of detection.
Specificity was tested directly. The AREG primer pair amplified AREG cDNA but gave no product on AREG-R or SELE cDNA, and all ten no-template controls were negative. Because AREG-R is the synonymously recoded trigger, this is a direct demonstration that the recoded sequence is a different RNA and not merely a different DNA construct. Each assay produced a single melt peak, consistent to within 0.32 °C across every well, which is one to three steps of the instrument's 0.108 °C reporting resolution. Replicate standard deviations were 0.012 to 0.282 Cq across all sample groups.




# NOT gate switches

An antisense NOT gate inverts a signal: when the matching RNA trigger is present, the gate blocks translation of the output, and the reporter goes dark. We placed the gate in the **5′ UTR of GFP**, between the 3′ end of the CMV promoter and the 5′ end of the

optimised GFP coding sequence. A trigger that pairs with the gate occludes the ribosome's path to the start codon.
Each switch therefore has to be inserted at one defined point in a plasmid that already carries its trigger. Rather than cut the vector with a restriction enzyme, we linearise it by **outward PCR** from those two points, which opens the plasmid exactly at the insertion site and leaves ends that the switch gBlocks match through their homology arms.

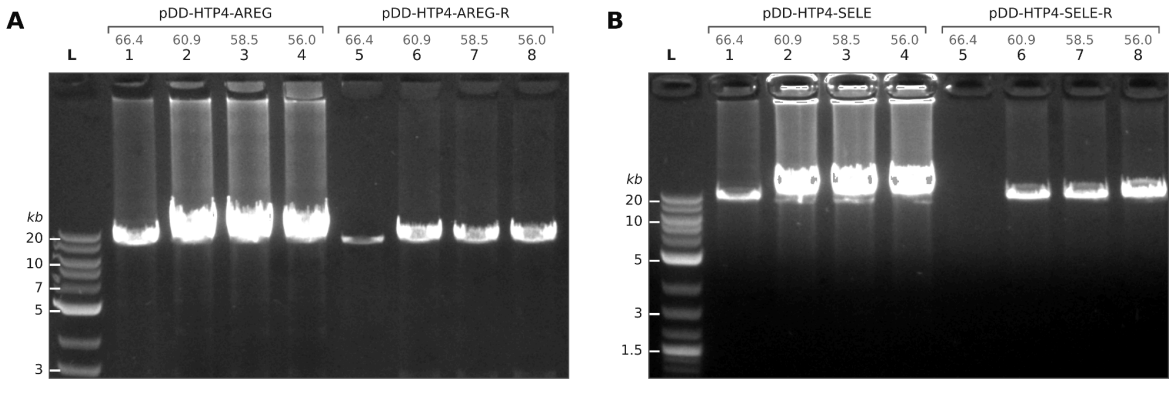
# Linearising the backbone

Two primers point outward from the insertion site, one back through the CMV promoter and one forward into GFP.

| Primer | Sequence (5′ to 3′) | Length | Tm |
|---|---|---|---|
| Rev-CMVpr-3end | GAGCTCTGCTTATATA GACCTCC | 23 nt | 57 °C |
| Fr-OptGFP-5end | GTGTCGAAAGGAGAA GAGTTATTTAC | 26 nt | 58 °C |

The two melting temperatures differ by 1 °C, which is the limit the Phanta Flash polymerase specifies for a primer pair. A larger mismatch would mean one primer annealing efficiently while the other does not, and on an 8 to 10 kb product that asymmetry shows up as low yield rather than as an obvious failure.
The same primer pair linearises all four plasmids, since both binding sites are in the shared backbone. The products differ in size because the trigger inserts do: roughly **8 kb** for the AREG and recoded AREG plasmids, roughly **10 kb** for the two The switch panel

**Backbone linearisation PCR, annealing-temperature gradient**




*expected linear product 8.0 kb* *expected linear product 9.8 kb*

Each construct was amplified at four annealing temperatures (66.4, 60.9, 58.5, 56.0 °C), loaded left to right within each group. Lane L, GeneRuler 1 kb Plus. Panels A and B are the two combs of a single gel, shown at identical magnification and identical contrast. A single linear brightness adjustment was applied uniformly to the whole image; no other processing was performed. Lanes 1 to 4 of both panels are saturated at this exposure.

Twenty switch designs were generated by the CERNAL pipeline, ten against each trigger. Five per trigger were taken forward, chosen to span the available length range rather than to cluster on one design.

| AREG switches | Length | SELE switches | Length |
|---|---|---|---|
| AREG-1 | 203 bp | SELE-1 | 183 bp |

| | | | |
|---|---|---|---|
| AREG-2 | 148 bp | SELE-2 | 148 bp |
| AREG-3 | 173 bp | SELE-4 | 203 bp |
| AREG-5 | 183 bp | SELE-6 | 128 bp |
| AREG-7 | 128 bp | SELE-7 | 128 bp |

All ten are short, 128 to 203 bp, and the longest is 1.6 times the shortest. Because Gibson assembly efficiency falls off below about 200 bp, and because a fixed mass of DNA means very different molar amounts across that range, each gBlock is diluted to a common **molar** working stock rather than a common concentration. Every assembly then receives the same number of insert molecules, at roughly three to four fold molar excess over the vector.

# Gibson Assembly

Assemblies were run as 5 µL Gibson reactions, 2.5 µL of 2× master mix and 2.5 µL of DNA, 45 to 60 min at 50 °C.
Backbone was used at 11.3 to 15.3 fmol per reaction and gBlock at 40 to 63 fmol, giving an insert to vector ratio of 2.6:1 to 5.6:1.

## Transformation and recovery

Each assembly was transformed into chemically competent cells and plated on LB with ampicillin. **Twelve of the twenty switch assemblies gave colonies.**

| Switch | In its own trigger | In the recoded trigger | Outcome |
|---|---|---|---|
| not_AREG-1 | recovered | no colonies | one side |
| not_AREG-2 | no colonies | recovered | one side |
| not_AREG-3 | recovered | recovered | **complete pair** |
| not_AREG-5 | recovered | no colonies | one side |
| not_AREG-7 | recovered | recovered | **complete pair** |
| not_SELE-1 | recovered | recovered | **complete pair** |
| not_SELE-2 | recovered | recovered | **complete pair** |
| not_SELE-4 | no colonies | no colonies | both sides |
| not_SELE-6 | no colonies | no colonies | both sides |
| not_SELE-7 | no colonies | recovered | one side |

The failures fall into two groups, and the split is informative because each switch sequence was assembled into two different backbones.

**Four designs failed on one side only.** not_AREG-1, not_AREG-2, not_AREG-5 and not_SELE-7 each produced clones in one backbone and nothing in the other. The switch sequence is identical in both, so a property of the switch cannot explain a one-sided loss. These are reaction or plating failures and should recover on a repeat.

**Toxicity hypothesis**

Toxicity is one explanation for the two both-sided failures, but it is not the first one to test. The switch sits in the 5′ UTR of a GFP cassette driven by a CMV promoter, which is not active in E. coli, so there is no obvious route to a toxic product. A failed or mis-synthesised gBlock produces the same result.

# Miniprep

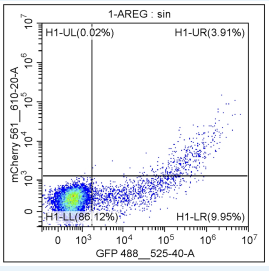
One colony per successful assembly was grown in 5 mL LB with ampicillin at 37 °C and 250 rpm, and glycerol stocks were taken before mini-prepping. Yields ran **81.4 to 329 ng/µL**.

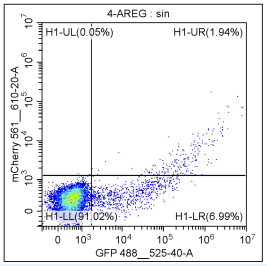
# Transfection

HEK293T cells were seeded on poly-L-lysine coated 96-well plates and transfected at a 1:3 DNA to reagent ratio, the conditions established in Cycle 1. Each construct went into one well. The four parent plasmids, which carry the trigger and both reporters but no switch, were transfected alongside as the unrepressed reference. Untransfected cells and a reagent-only well were included as negative controls.
Cells were read at 48 h.

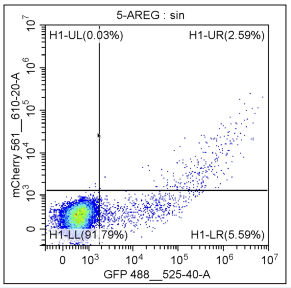
# Flow cytometry

18 acquisitions were taken on a CytoFLEX S, reading GFP on 488/525-40 and mCherry on 561/610-20. The negative controls set the thresholds: untransfected cells gave 0.24% GFP-positive and reagent-only 0.14%.

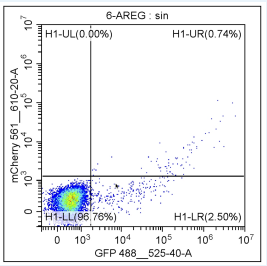
| Tube | Backbone | switch |
|---|---|---|
| 1 | AREG | not_AREG-1 |
|  | | |
| 4 | AREG-R | not_AREG-2 |



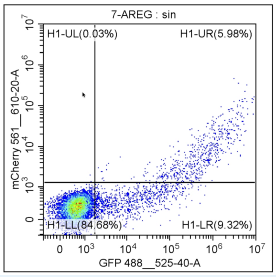

| | | |
|---|---|---|
| 5 | AREG | not_AREG-3 |



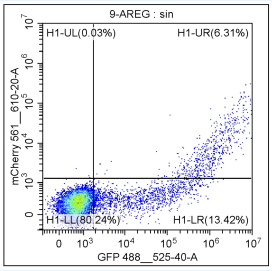

| | | |
|---|---|---|
| 6 | AREG-R | not_AREG-3 |



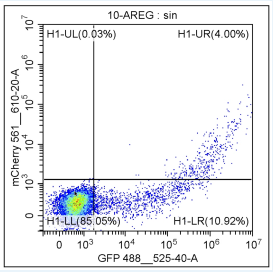

| | | |
|---|---|---|
| 7 | AREG | not_AREG-5 |



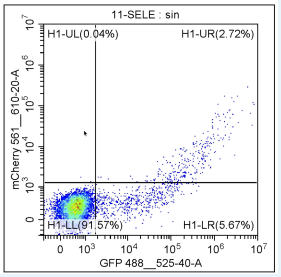

| | | |
|---|---|---|
| 9 | AREG | not_AREG-7 |



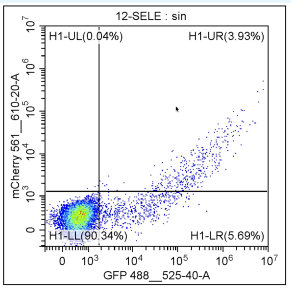

| | | |
|---|---|---|
| 10 | AREG-R | not_AREG-7 |



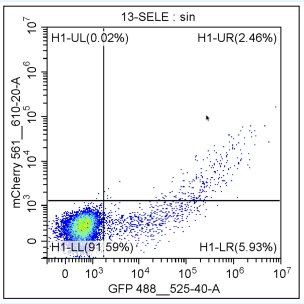

| | | |
|---|---|---|
| 11 | SELE | not_SELE-1 |



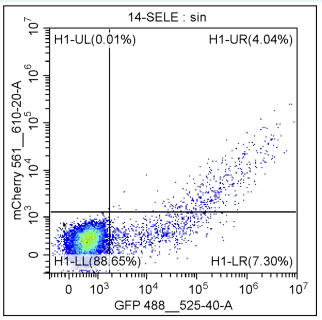

| | | |
|---|---|---|
| 12 | SELE-R | not_SELE-1 |



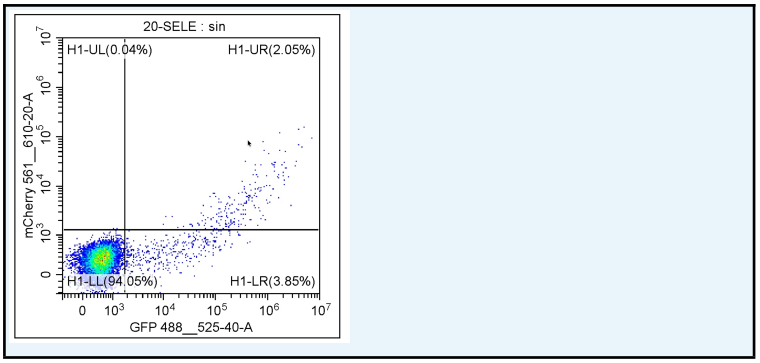

| | | |
|---|---|---|
| 13 | SELE | not_SELE-2 |

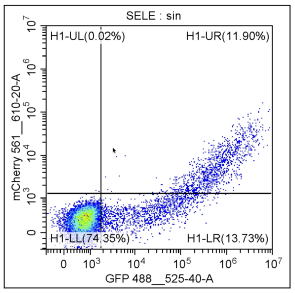


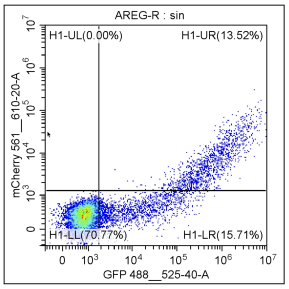

| | | |
|---|---|---|
| 14 | SELE-R | not_SELE-2 |

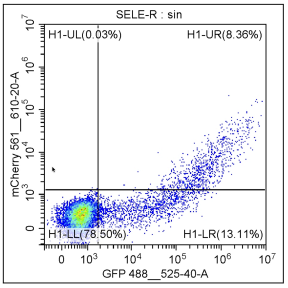


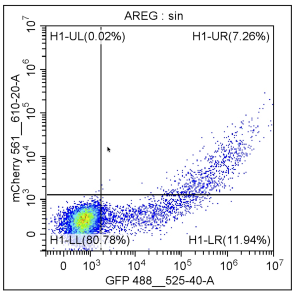

| | | |
|---|---|---|
| 20 | SELE-R | not_SELE-7 |



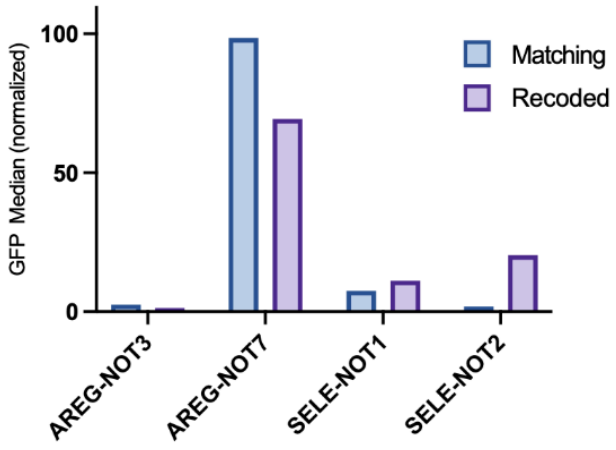

# Results

All twelve switch constructs give a lower fraction of GFP-bright cells than any of the four parents: 28.7 to 73.3% against 74.7 to 82.2%. Inserting the switch into the 5′ UTR costs reporter expression in every case, which is the mechanism the gate depends on.

GFP was summarised four ways in the mCherry-positive population.
Each statistic also has its own floor, taken as the spread across the four no-switch parents. Those four carry no switch and differ only in trigger sequence, so any difference between them is well-to-well variation rather than biology.

| | Before Gating | | | | | After Gating | | | | | | Normalized | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Num. of cells | GFP Mean | GFP SEM | GFP Median | GFP STD | Num. of cells | % Gated | GFP Mean | GFP SEM | GFP Median | GFP STD | GFP Mean | GFP SEM | GFP Median | GFP STD |
| **Cells** | 11513 | 537.73 | 4.2 | 495.8 | 450.32 | | | | | | | | | | |
| **Reagent only** | 12179 | 449.95 | 4.11 | 410.4 | 453.4 | | | | | | | | | | |
| **AREG** | 11487 | 70378.31 | 4262.55 | 729.1 | 456848.78 | 1474 | 12.83 | 468681.12 | 25488.34 | 119735.66 | 978566.31 | 79.94 | 2.48 | 56.3 | 95.33 |
| **AREG-R** | 11614 | 152571.98 | 6637.65 | 924.4 | 715328.56 | 2166 | 18.65 | 735202.75 | 29735.21 | 215026.31 | 1383886 | 82.78 | 1.81 | 65.71 | 84.35 |
| **SELE** | 11987 | 121205.8 | 5333.92 | 831.3 | 583985.19 | 1909 | 15.93 | 672406.75 | 26538.72 | 243315.8 | 1159532.62 | 100.48 | 2.31 | 79.54 | 100.75 |
| **SELE-R** | 12141 | 73547.66 | 4298.74 | 708.1 | 473662.25 | 1665 | 13.71 | 473661.03 | 24319.09 | 132239.2 | 992326 | 80.46 | 2.2 | 58.27 | 89.76 |
| **AREG-NOT1** | 12602 | 25783.76 | 1838.69 | 520.7 | 206408.69 | 1127 | 8.94 | 238127.56 | 17311.36 | 47596.3 | 581156.62 | 62.98 | 2.52 | 36.41 | 84.75 |
| **AREG-R-NOT2** | 12231 | 15124.71 | 1633.51 | 481.7 | 180656.31 | 776 | 6.34 | 204222.45 | 23909.73 | 1485.75 | 666048.12 | 46.73 | 2.8 | 1.86 | 77.97 |
| **AREG-NOT3** | 12183 | 22949.18 | 2827.9 | 523.7 | 312134.06 | 817 | 6.71 | 314229.38 | 40665.63 | 1976.4 | 1162354.25 | 41.63 | 2.38 | 2.56 | 67.9 |
| **AREG-R-NOT3** | 12240 | 4727.56 | 839.24 | 437.55 | 92848.66 | 443 | 3.62 | 107839.09 | 22513.21 | 921.3 | 473848.19 | 20.26 | 2.06 | 1.35 | 43.33 |
| **AREG-NOT5** | 12749 | 65225.53 | 4640.81 | 569 | 524000.38 | 1194 | 9.37 | 607517.31 | 40500.43 | 81786.91 | 1399464.25 | 65.52 | 2.56 | 37 | 88.56 |
| **AREG-NOT7** | 11335 | 141233.91 | 8436.02 | 741.1 | 898148.69 | 1237 | 10.91 | 1129268.75 | 62206.66 | 291756.81 | 2187871 | 149.25 | 5.18 | 98.54 | 182.34 |
| **AREG-R-NOT7** | 13414 | 58708.04 | 3989.95 | 557.05 | 462111.44 | 1090 | 8.13 | 672439.06 | 44883.41 | 124290.25 | 1481832.62 | 121.7 | 5 | 69.38 | 164.99 |
| **SELE-NOT1** | 12223 | 17745.89 | 1907.29 | 485 | 210865.31 | 799 | 6.54 | 232383.14 | 24595.88 | 8734 | 695241.56 | 38.1 | 2.03 | 7.5 | 57.26 |
| **SELE-R-NOT1** | 12036 | 22510.67 | 2057.47 | 547.9 | 225722.86 | 1051 | 8.73 | 213394.7 | 19340.32 | 12364.2 | 626996.19 | 28.6 | 1.24 | 11.13 | 40.27 |
| **SELE-NOT2** | 11388 | 14352.71 | 1343.2 | 563.55 | 143339.2 | 869 | 7.63 | 162894.55 | 16342.82 | 1413.6 | 481766.88 | 35.56 | 2.03 | 1.83 | 59.8 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **SELE-R-NOT 2** | 11478 | 33918.33 | 3630.25 | 592.25 | 388928.91 | 1025 | 8.93 | 299317.31 | 26270.46 | 32735.7 | 841065.25 | 42.36 | 2.05 | 20.33 | 65.49 |
| **SELE-R-NOT 7** | 13252 | 14932.88 | 2146.56 | 471.35 | 247106.06 | 732 | 5.52 | 191523.84 | 21810.07 | 1252.2 | 590082.38 | 34.28 | 2.42 | 1.69 | 65.49 |




Normalised GFP median for the four complete switch pairs. Each switch was assembled into the plasmid carrying its own trigger (matching) and into the plasmid carrying the synonymously recoded trigger (recoded). Values are GFP divided by mCherry per cell, medians of the mCherry-positive population, one well per construct. A functional NOT gate gives a lower value in the matching construct.

**not_SELE-2 gave a normalised GFP median of 1.83 with the matching trigger and 20.33 with the recoded one, an 11-fold difference.** not_SELE-1 gave 7.50 and 11.13. Both AREG pairs went the other way.

# Conclusions

- Both SELE switches are repressed by their matching trigger and stay on with the recorded one, which differs only by synonymous recording. The repression therefore tracks the trigger sequence.
- We created switches that can be assembled into the 5′ UTR of both the native and the recorded backbone, and twelve clones were recovered from twenty attempts.
- Two of the ten designs failed in both backbones, which localises the problem to those switch sequences rather than to the assembly.
- The inserted switch reduces reporter output in all twelve constructs, confirming that the sequence folds and interferes with translation.
- The dual-reporter readout needs a stable statistic. Percent positive and median brightness both fail on this population for reasons that can be demonstrated from the data, and percentage of gated cells that are bright does not.
- One of the four testable pairs shows trigger-dependent repression above the plate noise.

# Next Steps

Each construct was run in one well. not_SELE-2 and not_SELE-1 go into triplicate next. We are also repeating the four assemblies that gave colonies in only one backbone, and the AREG-R-NOT3 transfection, which would give eight testable pairs instead of four.

# References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