Broth and Glycerol Stock preparation

A. Lysogeny Broth

Materials:

- 25 g LB broth
- 1 L distilled water

Procedures:

- Add 25g LB broth to 1 litre distilled water
- Autoclave

B. LB Agar plates

Materials:

- 37 g LB Agar
- 1 Litre distilled water

Procedures:

- Add 37g LB Agar to 1 litre distilled water
- Autoclave
- Antibiotics were added before pouring plates (see Table 1)

C. Glycerol Stocks

Materials:

- 500µl Filtered glycerol solution (80%)
- 500µl overnight culture

Procedures:

- Add 500µl glycerol (80%) to 1.5ml Glycerol stocks tube
- Add 500µl overnight culture in LB
- Store at -80°C

Table 1. Antibiotic concentrations

Antibiotic	**Stock concentration**	**Working concentration**
Ampicillin	100mg/ml	100µg/ml
Chloramphenicol	25 mg/mL (dissolved in EtOH)	25µg/ml
Kanamycin	50 mg/mL	50µg/ml

Gel Electrophoresis

Materials:
Agarose Powder
TAE buffer
Gel mould
5-10 µL SybrSafe
Gel Tank
8-10 µL DNA ladder
DNA loading dye

Procedures:

1. Prepare 1% w/v solution of agarose powder in 1/10 TAE buffer (e.g. 1.0 g agarose powder in 100 mL buffer) using a conical flask
2. Heat the mixture until agarose is completely dissolved. Do not let the solution boil.
3. Pour the solution into a gel mould
4. Add 5-10 µL SybrSafe to the solution. Make sure there are no bubbles in the solution.
5. Allows the solution to set (approx 15-20 minutes)
6. Transfer the agarose gel to a tank, remove the comb and apply:
   - 8-10 µL of the DNA ladder
   - DNA samples with the corresponding amount of DNA loading dye (6X)
7. Run the gel for 45mins at 100V

Primer Design

1. Primers must be specified 5’ to 3’, left to right.
2. 18 to 25 nucleotides in length
3. GC content = 40%- 60%
4. 3’ ends = C or G, to promote binding (usually 2x, i.e. CC, CG GC GG; called “GC clamp”). GC base pairs have a stronger bond than AT pairs (3 hydrogen bonds vs 2).
5. 3’ end, (11 terminal nts) = should be an exact match to the templare DNA (proofreading DNA polymerases will degrade it otherwise).
6. When a restriction site sequence is added onto the 5’ end of a primer, an additional 3-6 nts (a leader sequence) are needed to ensure efficient cutting.
7. Avoid runs of 4 or more of any one base, or dinucleotides repeats (ACCCC or ATATATAT); this can cause mispriming.
8. Check for secondary structures: primer dimers self-dimers and hetero dimers, and hairpins.
    - ¶ Use IDT oligo analyzer to detect secondary structures and hairpins. https://www.idtdna.com/calc/analyzer
    - ¶ The delta G value for dimer analysis should be between 0- -5 kcal/mole for optimal design. More negative values will affect PCR reactions.
    - ¶ For hairpins, the melting temperature, Tm should be lower than the annealing temperature and no greater 50 degrees Celsius
9. Primer pairs should have similar Tm with a maximum difference of 5 degrees Celsius.
10. Primers should not be complementary to each other.
11. Use snapgene or ncbiprimer blast(www.ncbi.nlm.nih.gov/tools/primer-blast/) or UCSC in-silico PCR (genome.ucsc.edu) to verify that primer only anneal in one place.
12. Gradient PCR, 2- Step PCR, overlap-extension PCR, Gibson Assenbly/ HiFi, Golden Gate, 3A assembly.

Mini-prep Protocol

Materials:

1. Re-suspension buffer
2. Lysis solution
3. Neutralization solution
4. Wash solution
5. Elution buffer
6. Overnight culture
7. Eppendorf tubes
8. Benchtop centrifuge

Procedures:

1. Prepare overnight culture
2. Start with 2 mL of overnight culture
3. Centrifuge at 12500rpm for 5 mins
4. Discard supernatant and re-suspend with 250µL re-suspension buffer
5. Add 250µL lysis buffer and mix gently by inverting tubes 4-6 times
6. Add 350µL neutralization buffer and invert tubes immediately.
7. Centrifuge at 12500rpm for 5 mins
8. Transfer supernatant into spin-column
9. Add 750µL wash buffer and centrifuge for 1 mins at 12500 rpm.
10. Discard the flow-through and repeat step 9 and 10 again
11. Centrifuge 1 more minute to get rid of ethanol
12. Discard the tubes at the bottom of column and transfer it to Eppendorf tube
13. Add 30-50µl elution buffer and incubate at benchtop for 2 mins
14. Centrifuge for 1 mins
15. Collect the tubes with plasmid in it and store in -20˚C freezer

Overnight Culture Preparation

Materials:

1. 10 ml LB media
2. Falcon Tube
3. Antibiotics (see Table 1)
4. Inoculation Loops

Procedures:

1. Add 10 ml LB media into Falcon tube
2. Add antibiotic at the appropriate final concentration
3. Use an inoculating loop, pick a colony and touch the culture by dipping
4. Seal the tube and incubate overnight at 37˚C shaking incubator

Table 1. Antibiotic concentrations

Antibiotic	**Stock concentration**	**Working concentration**
Ampicillin	100mg/ml	100µg/ml
Chloramphenicol	25 mg/mL (dissolved in EtOH)	25µg/ml
Kanamycin	50 mg/mL	50µg/ml

Transformation

Materials:

1. Ice box
2. 42˚C water bath
3. 37˚C water bath
4. 37˚C shaking incubator
5. Eppendorf tubes
6. NEB10ß or DH5α competent cells

Procedures:

1. Thaw cells on ice
2. Mix 1 - 5 µl of DNA (usually 10 pg - 100 ng) into 20-50 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
3. Incubate the competent cell/DNA mixture on ice for 20-30 mins.
4. Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 secs (45 secs is usually ideal, but this varies depending on the competent cells you are using).
5. Put the tubes back on ice for 2 min.
6. Add 250-1,000 µl LB or SOC media (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 45 min.
7. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
8. Incubate plates at 37°C overnight.

Part A. Testing expression levels of fusion proteins:

1. Intimin'-SpyTag
2. Intimin'-SpyCatcher

Experiments in brief:

1. Two cell lines expressing either fusion protein will be combined and will measure sedimentation levels or using a particle sizer
    a. Wild type + wild type - measure sedimentation
    b. Wild type + SpyTag/Catcher - measure sedimentation (after DNAcome)
2. Visualize protein localization using GFP tagged SpyTag or SpyCatcher added externally onto cells expressing surface binding partner (cell-protein interaction)
3. Visualize expression levels of each fusion protein using SDS PAGE gel
    a. Run wild-types through the SDS PAGE.
4. His tag purification of complex formed

Protein Expression protocol

Day n-1, Late afternoon

Set up starter culture:
10ml medium/antibiotic
1 colony from plate/glycerol stock
37 oC o/n

Day 1

Subculture o/n culture into fresh medium/antibiotic.
Volume: more than enough to split into later cultures.
Dilution: Add o/n culture to 1-2% final volume.
37 oC, 2h Measure OD600
Blank w clean medium.

Induce if mid-log phase. (Log phase OD600 = 0.4-0.8. Mid-log phase is around 0.6.) (If not yet log phase/mid-log phase, return culture to incubator, check again in 20-30mins.)

Split culture into -+ induction (or whatever conditions you're trying). (Uninduced control is pretty important.)
Add inducer to relevant concentration (eg. IPTG to 1 mM).
Incubate: 37 oC, 2-3 h or 30 oC, 3-6 h or 16-25 oC o/n

Harvest cells:
Take final OD.
Pellet 2.5 OD cells in cooled microfuge (usually around 1-2ml culture). (4min, 8,000 xg, 4oC)
- wash in 500 µl cold buffer*, re-pellet
- resuspend in lysis buffer*
*Buffers to use here will depend on application. You don't want the wash buffer to be a lysis buffer, so Tris-Cl of ~ pH 7.5 w NaCl between 50-150 mM is ideal.

Lysis buffer: can be like wash buffer if sonicating. Can also add reagents for lysis, eg. detergents like Tween or Triton. Most commonly I add lysozyme to 1mg/ml final concentration and incubate at for 30 oC, 20min.

50 mM Tris-HCl pH 7.5
100 mM NaCl
1 mM DTT (for intracellular proteins)
5% glycerol (possibly)

I keep the final buffer to 250 µl usually. This means you'll have 2.5OD cells/250 µl = 0.01 OD/µl, which is a good concentration, as 10 µl of this (0.1OD) is a decent # cells and a decent amount of proteins to load onto SDS-PAGE.

Lysis either:
(1) sonication: cup horn sonicator, settings 100% amplitude, 2min, 30s on/30s off.
(2) lysozyme (see above) - typically followed by sonication as well.
(3) can freeze pellet after washing (see above), and on thawing, either do sonication or lysozyme method.
Separation of fractions: spin in cooled microfuge: 30' at 16,000 xg, 4oC pellet will contain **insoluble proteins**, membrane fractions. **Soluble** material can be transferred to new eppie. pellet can be resuspended in 250 µl 1X LB.

Prep samples for gel: Soluble: 15 µl sample + 15 µl 2xLB [= 30 µl, of which to load 20 µl on gel] boil 95oC, 5min
Insoluble: boil whole 250 µl as above.

Day 2

Run SDS-PAGE gels to determine whether your protein was expressed.
Typically, MW ladder (NEB colour prestained/broad range, 5 µl), soluble fractions, insoluble fractions. Soluble: load 20 (of 1:1 sample:2xLB), Insoluble: load 5 µl (of sample in 1xLB).
*Insoluble fractions run better if they've been passed through a syringe to break up DNA.

Typically: 80-100 V until samples get through stacking gel, then 120V+. This takes ~2h total. (Technically possible to run it much faster, resolution is very poor though.)
Stain: instant blue, 30min RT on rocker. Destain in dH2O, 30min or o/n.
Capture on Licor

Part B. Testing covalent interaction between fusion proteins in vitro

SDS-PAGE to detect amide bond formation between SpyTag-fusion protein and SpyCatcher

Proteins mixed at 10uM in PBS pH7.4 at 24*C for 3hr (quantifications performed in triplicate)
Stop reaction by heating samples in SDS loading buffer on a Bio-Rad C1000 thermal cycler at 95 °C for 7 min.
SDS-PAGE on 14% polyacrylamide gels, at 200 V for approximately 1 h.
Gels stained with Instant Blue Coomassie stain (Triple Red Ltd.) and band intensities were quantified using a Gel Doc XR imager and Image Lab 3.0 software (Bio-Rad).

Reactions for pH-dependence: Mixing 10 μM of each protein in 40 mM Na2HPO4 with 20 mM citric acid pH 7.0 (phosphate-citrate) for the indicated time at 25 °C, or at the indicated temperature and pH. (PBS alone would not enable proper buffering over the pH range explored.)
For determining temperature dependence: all reactions were incubated in a Bio-Rad C1000 thermal cycler at 4, 25, and 37 °C with a heated lid to prevent evaporation.
To calculate the rate constant, SpyTag-MBP and SpyCatcher at 10 μM were mixed in triplicate in phosphate-citrate and incubated at 25 °C for 1, 3, or 5 min, in the linear part of the reaction. Samples were then heated to 95 °C for 7 min in SDS loading buffer and analyzed on 14% SDS-PAGE with Coomassie staining. Unreacted SpyCatcher concentration was quantified from band intensity as above. 1⁄[unreacted Spy Catcher] was plotted against time and a straight line, whose gradient corresponds to the second order rate constant, was fitted using the "LINEST" linear least squares curve-fitting routine in Excel. The units were converted from μM−1 min−1 to M−1 s−1.

→ could include GFP to see in vivo localization of fusion proteins

Part C. Testing covalent interaction between fusion proteins

- Combine cultures, one expressing SpyTag fusion protein (that had the highest expression levels) with another culture expressing the SpyCatcher fusion protein (which had the highest expression levels)
- Repeat protein expression protocol
- Isolate protein extracts using His column
  → ensure His tag is on SpyCatcher

Cell aggregation:
http://www.pnas.org/content/100/23/13259.full

Microscopy and Data Acquisition. To record the motion of individual cells in the clusters, the slides were left undisturbed for ≈20 min on the microscope stage, which was found to be the optimal time required to form tight clusters that did not drift. Images were acquired within the window of 20–40 min because after 40 min many cells lost motility and the clusters had a tendency to disperse. This result is probably caused by exhaustion of oxygen by bacterial respiration. Cells grown in LB medium typically loose motility under a coverslip after ≈20 min. The cluster size in LB medium is comparable to that observed in minimal media (M9 glycerol). Both dark field and fluorescence microscopy were used to characterize the macroscopic cluster properties and the motility of single cells. Images were acquired at 15 frames/s by using a Nikon E800 microscope equipped with a cooled CCD camera (CoolSNAP$_{HQ}$, Roper Scientific, Duluth, GA).
Data Analysis. Cells were tracked by using Metamorph (Universal Imaging Systems), which yielded the coordinates of each fluorescent cell for every frame of the video. Cells that left the field of view were not tracked. These data were then analyzed by using a tumble detection algorithm similar to Alon *et al.* (19) implemented in MATLAB (Mathworks, Natick, MA). Cells that swum at <75% of the mean speed were discarded from the data set. Success of the program was verified by visual examination of recordings and was found to be 90% accurate. The errors were largely due to cells that did not slow down while changing direction. It is possible that these may not in fact be tumble events but instead collisions with other cells or with the slide or coverslip. The center of a cluster was determined by averaging dark field images acquired over 6.5 s. In total, from the trajectories of 32 individual cells expressing GFP found in 28 clusters, 97 tumble events were registered. To determine the cell density profile, 19 clusters consisting only of cells expressing GFP were analyzed. The mean swimming speed of cells was determined to be 27 μm/s, which is the projected 2D speed. The actual speed will be a slightly greater because the system is only quasi-2D; i.e., the height of the system was ≈40 μm, which is approximately one-fifth of the typical cluster diameter.

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0107445
Fluorescence microscopy
For the selection lines derived from *E. coli* MGAY, when the cells reached an absorbance 0.2 (600 nm), they were placed on a slide that was layered with a 1.5% agarose pad and covered with a cover slip to immobilize the cells. Observations were made using differential interference contrast (DIC) and fluorescence imaging on Zeiss Axioimager M-1 upright microscope (100X/1.40 Oil DIC M27) with a color digital camera controlled by Axio software 4.8. Images both DIC/fluorescence were taken using exposure time of 30 millisecond illumination. The resulting images were processed and analyzed using Image J 1.44c software [25]. The fluorescence image alone was used for quantification of fluorescence intensity reflecting aggregate size whereas composite image formed by merging DIC and fluorescence was used to determine the location of protein aggregates in a cell.

Part D: Light induction

Introduce construct of best fusion protein partners in plasmid downstream of the light induction control promoter

1. Seed cultures from freshly transformed plates and grow overnight in 5 ml of LB medium supplemented with appropriate antibiotics at 37°C with shaking (225 rpm). Where specified, *N*-(3-oxo-hexanoyl)-l-HSL was added to the cultures at the indicated concentrations.
2. Overnight cultures were diluted into 5 ml of fresh pre-warmed M9 medium [(12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl), 1 M $MgSO_4$, 1 M $CaCl_2$, 0.2% (w/v) casamino acids and 20 mM glucose as a sole carbon source. As appropriate, antibiotics] for 2–3 h exponential outgrowth at 37°C with shaking (225 rpm).

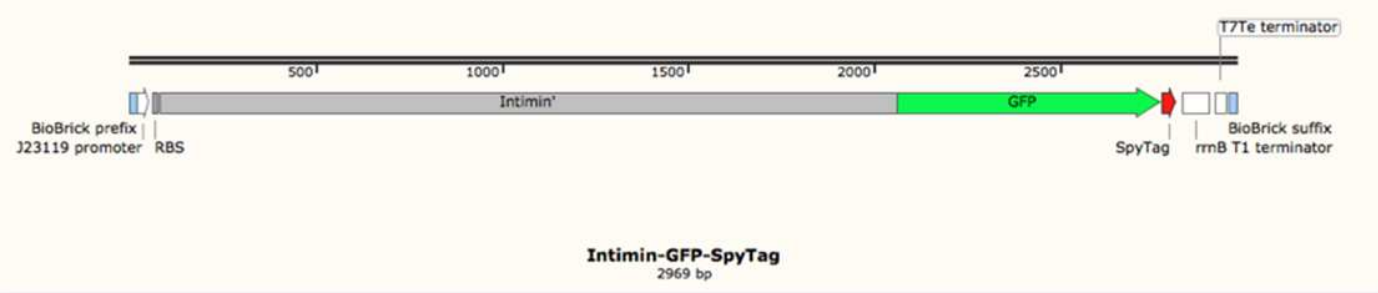
3. Cultures aliquoted (0.5 ml) into 0.5 ml of pre-warmed M9 medium in triplicates of total 1 ml volume in a flat-bottom 12-well microplate (Nunc™). The microplate was incubated at 37°C with shaking (120 rpm) in a mini shaker incubator with illumination or kept in dark (wrapped in black cloth covering all edges) between each cycle of measurements.
4. Cells are illuminated using a custom built 3 × 4 LED blue light panel (465 nm) with adjustable ON/OFF pulsing and intensity for individual wells.

IF WE SOMEHOW HAVE GFP expression as well, we could observe directly junction between cells that have adhered, fluoresce

Time series OD (600 nm) and fluorescence (GFP: excitation 485 nm, emission 528 nm and RFP: excitation 540 nm, emission 600 nm) were read using Synergy™ HTX Multi-Mode Microplate Reader (BioTek). In all experiments, auto-fluorescence was measured using a negative control strain lacking the fluorescence reporter. The fluorescence/OD_{600} (Fluo/OD_{600}) at a specific time for a sample culture was determined after subtracting from each of the technical triplicate readings of the negative control cultures (fluorescence free) at the same time. The fluorescence synthesis rate (Fluo.$OD_{600}{}^{-1}$/min) of any sample at time t, was calculated by taking the difference of Fluo/OD_{600} values from two time points and dividing the result by the time interval δt. Normalization of Fluo/OD_{600} values was calculated into a new arbitrary range (min = 0) to (max = 1) with the observed original dataset min and max values.

Otherwise we would just perform protein expression assays post light induction vs no light induction and His tag column

1. Intimin GFP SpyTag

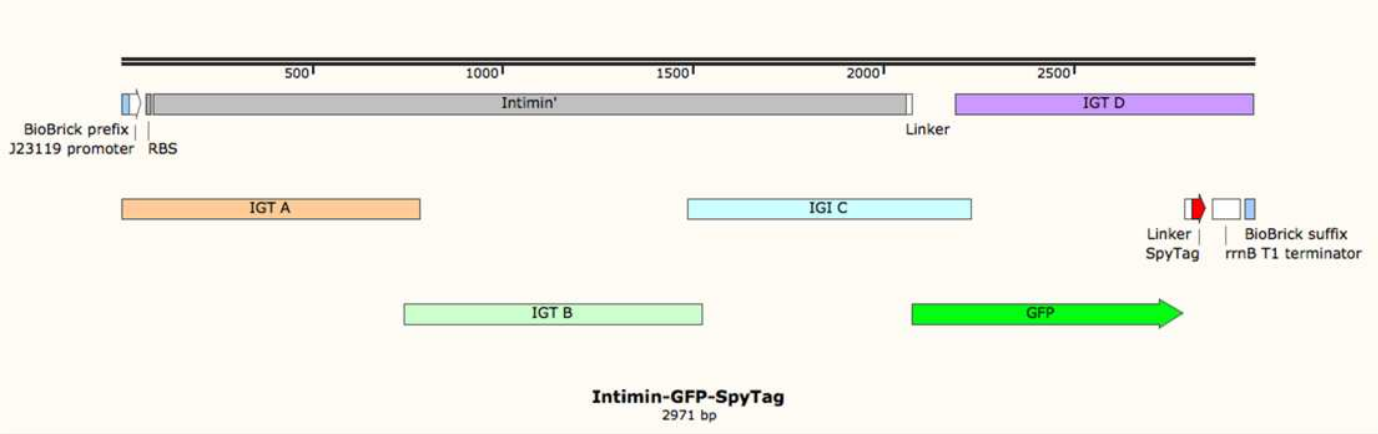


FusionGFPSpy: PROM-RBS-Intimin'-GFP-SpyTag-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgattacccatggctgctatacccgcacc
cgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagctttgcgaacggcgaaaactattttaaactg
ggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggcgaaaccgtggcggatctgagcaaaagccaggatattaac
ctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgccgggccagcagattattctgccgctgaaaaaactgccgttt
gaatatagcgcgctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtggcgggccataccaacaaactgaccaaaatgagcccggatgtgaccaaa
agcaacatgaccgatgataaagcgctgaactatgcggcgcagcaggcggcgagcctgggcagccagctgcaAagccgcagcctgaacggcgattatgcgaaaga
taccgcgctgggcattgcgggcaaccaggcgagcagccagctgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttga
tggcagcagcctggattttctgctgccgttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgctttaccgcgaacctgggcgcg
ggccagcgctttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctgggcattggcggcgaatattggcgcgattat
tttaaaagcagcgtgaacggctattttcgcatgagcggctggcatgaaagctataacaaaaaagattatgatgaacgcccggcgaacggctttgatattcgctttaacggc
tatctgccgagctatccggcgctgggcgcgaaactgatttatgaacagtattatggcgataacgtggcgctgtttaacagcgataaactgcaAagcaacccgggcgcgg
cgaccgtgggcgtgaactataccccgattccgctggtgaccatgggcattgattatcgccatggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgctatc
agtttgataaaagctggagccagcagattgaaccgcagtatgtgaacgaactgcgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattct
ggaatataaaaaacaggatattctgagcctgaacattccgcatgatattaacggcaccgaacatagcacccagaaaattcagctgattgtgaaaagcaaatatggcctg
gatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcatagcggcagccagagcgcgcaggattatcaggcgattctgccggcgtatgtg
cagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcgcaacggcaacagcagcaacaacgtgcagctgaccattaccgtgctgagcaacggccag
gtggtggatcaggtgggcgtgaccgattttaccgcggataaaaccagcgcgaaagcggataacgcggataccattacctataccgcgaccgtgaaaaaaaacggcgt
ggcgcaggcgaacgtgccggtgagctttaacattgtgagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccc
tgaaaagcagcaccccgggccaggtggtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgag
cggcagcggcggcagcggcAtgcgtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaatttttctgtcagtgga
gagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcactactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaat
gctttgcgagatacccagatcatatgaaacagcatgacttttttcaagagtgccatgcccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactaca
agacacgtgctgaagtcaagtttgaaggtgataccctgttaatagaatcgagttaaaaggtattgattttaaagaagatggaaacattcttggacacaaattggaatacaa
ctataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattagacacaacattgaagatggaagcgttcaactagcaga
ccattatcaacaaaatactccaattggcgatggccctgtccttttaccagacaaccattacctgtccacacaatctgccctttcgaaagatcccaacgaaaagagagacca
catggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaactatacaaaggcagcggcggcagcggcgcgcatattgtgatggtggatgcgtataa
accgaccaaatactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacac
tggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
Intimin'
GFP
Linker
SpyTag

Assembly through Gibson or in-fusion assembly



IGTA

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgattacccatggctgcta
tacccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagctttgcgaacg
gcgaaaactattttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggcgaaaccgtgg
cggatctgagcaaaagccaggatattaacctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgcc
gggccagcagattattctgccgctgaaaaaactgccgtttgaatatagcgcgctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtgg
cgggccataccaacaaactgaccaaaatgagcccggatgtgaccaaaagcaacatgaccgatgataaagcgctgaactatgcggcgcagcaggc
ggcgagcctgggcagccagctgcaAagccgcagcctgaacggcgattatgcgaaagataccgcgctgggcattgcgggcaaccaggcgagcagc
cagctgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttgatggcagcagcctggattttctgctgccgttt

IGTB

aacaactttgatggcagcagcctggattttctgctgccgtttt atgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgctt
taccgcgaacctgggcgcgggccagcgctttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcct
gggcattggcggcgaatattggcgcgattattttaaaagcagcgtgaacggctattttcgcatgagcggctggcatgaaagctataacaaaaaagattat
gatgaacgcccggcgaacggctttgatattcgctttaacggctatctgccgagctatccggcgctgggcgcgaaactgatttatgaacagtattatggcgat
aacgtggcgctgtttaacagcgataaactgcaAagcaacccgggcgcggcgaccgtgggcgtgaactataccccgattccgctggtgaccatgggca
ttgattatcgccatggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgctatcagtttgataaaagctggagccagcagattgaaccgcag
tatgtgaacgaactgcgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattctggaatataaaaaacaggatattctgagc
ctgaacattccgcatgatattaacggcaccgaacatagcacccagaaaattcagctgattgtgaaaagcaaatatggc

IGTC

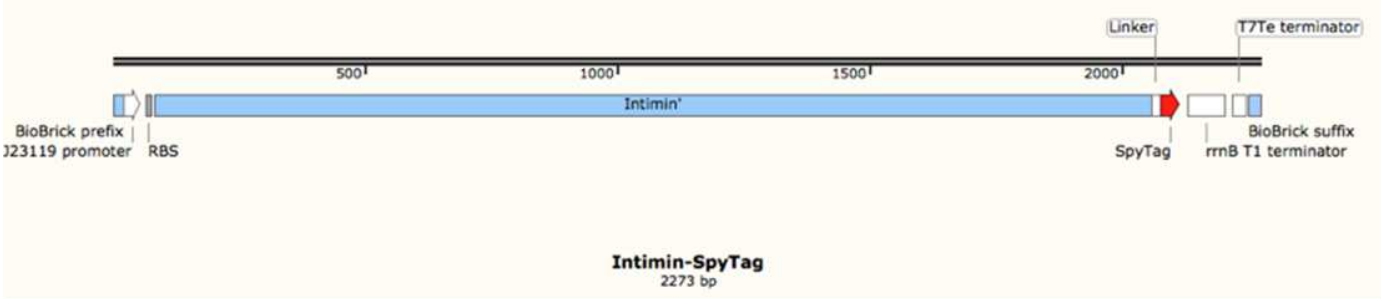
cacccagaaaattcagctgattgtgaaaagcaaatatggcctggatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagc
atagcggcagccagagcgcgcaggattatcaggcgattctgccggcgtatgtgcagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgat
cgcaacggcaacagcagcaacaacgtgcagctgaccattaccgtgctgagcaacggccaggtggtggatcaggtgggcgtgaccgattttaccgcgg
ataaaaccagcgcgaaagcggataacgcggataccattacctataccgcgaccgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgag
ctttaacattgtgagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccctgaaaagcagcacc
ccgggccaggtggtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgagcggc
agcggcggcagcggcAtgcgtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtca
gtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcactactgga

IGTD

catacggaaaacttacccttaaatttatttgcactactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgag
atacccagatcatatgaaacagcatgacttttcaagagtgccatgcccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactaca
agacacgtgctgaagtcaagtttgaaggtgatacccttgttaatagaatcgagttaaaaggtattgattttaaagaagatggaaacattcttggacacaaatt
ggaatacaactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattagacacaacattgaagatg

gaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtcctttaccagacaaccattacctgtccacacaatctgccctttc
gaaagatcccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaactatacaaaggcagcg
gcggcagcggcgcgcatattgtgatggtggatgcgtataaaccgaccaaatactagagccaggcatcaaataaaacgaaaggctcagtcgaaagac
tgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacacttactagtagcggccgctgca

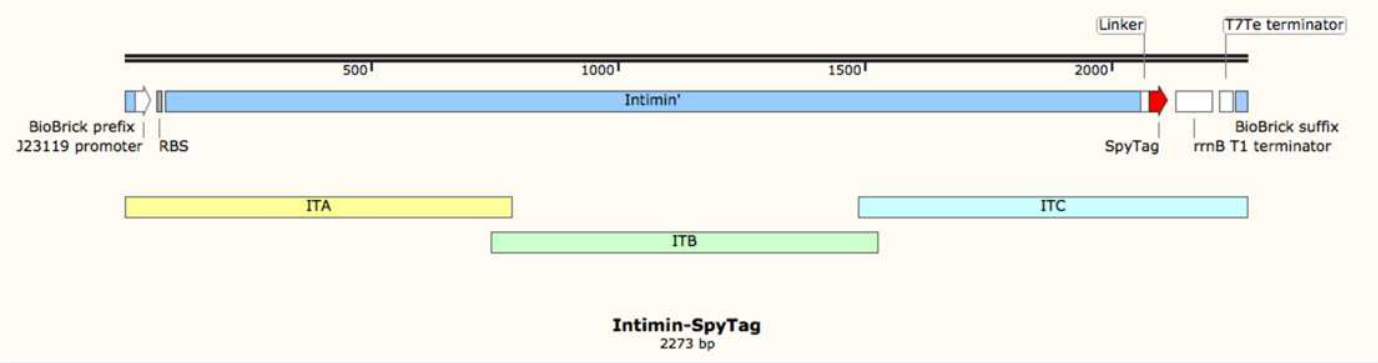
2. Intimin SpyTag



FusionSpy1: PROM-RBS-Intimin'-SpyTag-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgattacccatggctgctatacccgcacc
cgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagctttgcgaacggcgaaaactattttaaactg
ggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggcgaaaccgtggcggatctgagcaaaagccaggatattaac
ctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgccgggccagcagattattctgccgctgaaaaaactgccgttt
gaatatagcgcgctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtggcgggccataccaacaaactgaccaaaatgagcccggatgtgaccaaa
agcaacatgaccgatgataaagcgctgaactatgcggcgcagcaggcggcgagcctgggcagccagctgcaAagccgcagcctgaacggcgattatgcgaaaga
taccgcgctgggcattgcgggcaaccaggcgagcagccagctgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttga
tggcagcagcctggattttctgctgccgttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgctttaccgcgaacctgggcgcg
ggccagcgcttttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctgggcattggcggcgaatattggcgcgattat
tttaaaagcagcgtgaacggctattttcgcatgagcggctggcatgaaagctataacaaaaaagattatgatgaacgcccggcgaacggctttgatattcgctttaacggc
tatctgccgagctatccggcgctgggcgcgaaactgatttatgaacagtattatggcgataacgtggcgctgtttaacagcgataaactgcaAagcaacccgggcgcgg
cgaccgtgggcgtgaactataccccgattccgctggtgaccatgggcattgattatcgccatggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgctatc
agtttgataaaagctggagccagcagattgaaccgcagtatgtgaacgaactgcgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattct
ggaatataaaaaacaggatattctgagcctgaacattccgcatgatattaacggcaccgaacatagcacccagaaaattcagctgattgtgaaaagcaaatatggcctg
gatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcatagcggcagccagagcgcgcaggattatcaggcgattctgccggcgtatgtg
cagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcgcaacggcaacagcagcaacaacgtgcagctgaccattaccgtgctgagcaacggccag
gtggtggatcaggtgggcgtgaccgattttaccgcggataaaaccagcgcgaaagcggataacgcggataccattacctataccgcgaccgtgaaaaaaaacggcgt
ggcgcaggcgaacgtgccggtgagctttaacattgtgagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccc
tgaaaagcagcaccccgggccaggtggtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttttgatcagaccaaagcgag
cggcagcggcggcagcggcgcgcatattgtgatggtggatgcgtataaaccgaccaaatactagagccaggcatcaaataaaacgaaaggctcagtcgaaagact
gggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
Intimin'
Linker
SpyTag



ITA

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgattacccatggctgcta
tacccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagctttgcgaacg
gcgaaaactattttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggcgaaaccgtgg
cggatctgagcaaaagccaggatattaacctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgcc
gggccagcagattattctgccgctgaaaaaactgccgtttgaatatagcgcgctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtgg
cgggccataccaacaaactgaccaaaatgagcccggatgtgaccaaaagcaacatgaccgatgataaagcgctgaactatgcggcgcagcaggc
ggcgagcctgggcagccagctgcaAagccgcagcctgaacggcgattatgcgaaagataccgcgctgggcattgcgggcaaccaggcgagcagc
cagctgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttgatggcagcagcctggattttctgctgccgttt

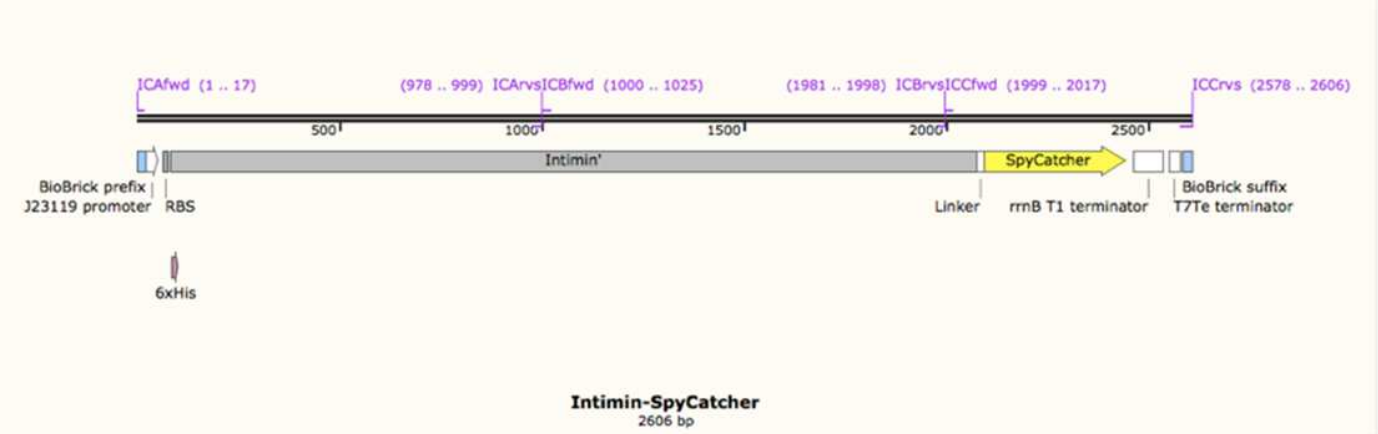
ITB

acaactttgatggcagcagcctggattttctgctgccgtttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgcttt
accgcgaacctgggcgcgggccagcgctttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctg
ggcattggcggcgaatattggcgcgattattttaaaagcagcgtgaacggctattttcgcatgagcggctggcatgaaagctataacaaaaaagattatg
atgaacgcccggcgaacggctttgatattcgctttaacggctatctgccgagctatccggcgctgggcgcgaaactgatttatgaacagtattatggcgata
acgtggcgctgtttaacagcgataaactgcaAagcaacccgggcgcggcgaccgtgggcgtgaactataccccgattccgctggtgaccatgggcatt
gattatcgccatggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgctatcagtttgataaaagctggagccagcagattgaaccgcagt
atgtgaacgaactgcgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattctggaatataaaaaacaggatattctgagcc
tgaacattccgcatgatattaacggcaccgaacatagcaccagaaaattcagctgattgtgaaaagcaaatatggcctg

ITC

ccagaaaattcagctgattgtgaaaagcaaatatggcctggatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcata
gcggcagccagagcgcgcaggattatcaggcgattctgccggcgtatgtgcagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcg
caacggcaacagcagcaacaacgtgcagctgaccattaccgtgctgagcaacggccaggtggtggatcaggtgggcgtgaccgattttaccgcggat
aaaaccagcgcgaaagcggataacgcggataccattacctataccgcgaccgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgagctt
taacattgtgagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccctgaaaagcagcacccc
gggccaggtggtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgagcggcag
cggcggcagcggcgcgcatattgtgatggtggatgcgtataaaccgaccaaatactagagccaggcatcaaataaaacgaaaggctcagtcgaaag
actgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccg
ctgcag

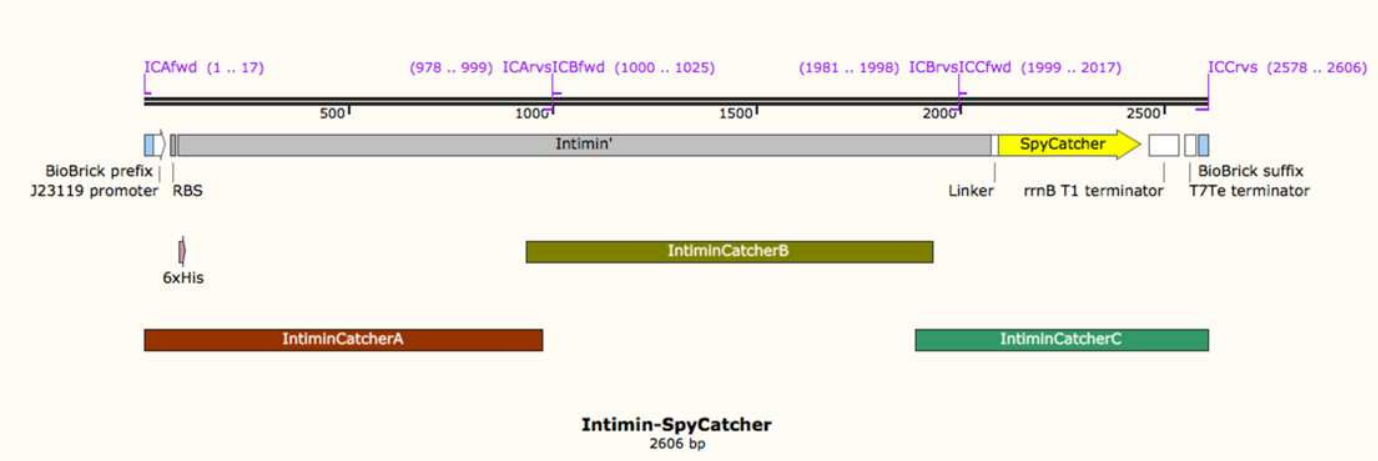
3. Intimin SpyCatcher



FusionSpy2: PROM-RBS-V5tag-Intimin'-SpyCatcher-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgCACCATCACCATCACCAT
attacccatggctgctatacccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagctttg
cgaacggcgaaaactattttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggcgaaaccgtggcgg
atctgagcaaaagccaggatattaacctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgccgggccagcagat
tattctgccgctgaaaaaactgccgtttgaatatagcgcgctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtggcgggccataccaacaaactgac
caaaatgagcccggatgtgaccaaaagcaacatgaccgatgataaagcgctgaactatgcggcgcagcaggcggcgagcctgggcagccagctgcaAagccgc
agcctgaacggcgattatgcgaaagataccgcgctgggcattgcgggcaaccaggcgagcagccagctgcaAgcgtggctgcaAcattatggcaccgcggaagtg
aacctgcaAagcggcaacaactttgatggcagcagcctggattttctgctgccgttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgata
gccgctttaccgcgaacctgggcgcgggccagcgctttttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctggg
cattggcggcgaatattggcgcgattattttaaaagcagcgtgaacggctattttcgcatgagcggctggcatgaaagctataacaaaaaagattatgatgaacgcccgg
cgaacggctttgatattcgctttaacggctatctgccgagctatccggcgctgggcgcgaaactgatttatgaacagtattatggcgataacgtggcgctgtttaacagcgat
aaactgcaAagcaacccgggcgcggcgaccgtgggcgtgaactatac cccgattccgctggtgaccatgggcattgattatcgccatggcaccggcaacgaaaacg
atctgctgtatagcatgcagtttcgctatcagtttgataaaagctggagccagcagattgaaccgcagtatgtgaacgaactgcgcaccctgagcggcagccgctatgatc
tggtgcagcgcaacaacaacattattctggaatataaaaaacaggatattctgagcctgaacattccgcatgatattaacggcaccgaacatagcacccagaaaattca
gctgattgtgaaaagcaaatatggcctggatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcatagcggcagccagagcgcgcagg
attatcaggcgattctgccggcgtatgtgcagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcgcaacggcaacagcagcaacaacgtgcagctg
accattaccgtgctgagcaacggccaggtggtggatcaggtgggcgtgaccgattttaccgcggataaaaccagcgcgaaagcggataacgcggataccattacctat
accgcgaccgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgagctttaacattgtgagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccga
tgcgaacggcaaagcgaccgtgaccctgaaaagcagcaccccgggccaggtggtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgc
ggtgatttttttgatcagaccaaagcgagcggcagcggcggcagcggcatggcgggcgtggataccctgagcggcctgagcagcgaacagggccagagcggcgat
atgaccattgaagaagatagcgcgacccatattaaatttagcaaacgcgatgaagatggcaaagaactggcgggcgcgaccatggaactgcgcgatagcagcggc
aaaaccattagcacctggattagcgatggccaggtgaaagatttttatctgtatccgggcaaatatacctttgtggaaaccgcggcgccggatggctatgaagtggcgac
cgcgattacctttaccgtgaacgaacagggccaggtgaccgtgaacggcaaagcgaccaaaggcgatgcgcatattaccggctactagagccaggcatcaaataaa
acgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatact
agtagcggccgctgcag

Prefix/Suffix
Intimin'
Linker
SpyCatcher
6xHis



IntiminCatcherA (ICA)

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgCACCATCACCA
TCACCATattacccatggctgctatacccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttta
tgtgaaccagaacagctttgcgaacggcgaaaactatttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttata
ccctgaaaaccggcgaaaccgtggcggatctgagcaaaagccaggatattaacctgagcaccatttggagcctgaacaaacatctgtatagcagcga
aagcgaaatgatgaaagcggcgccgggccagcagattattctgccgctgaaaaaactgccgtttgaatatagcgcgctgccgctgctgggcagcgcg
ccgctggtggcggcgggcggcgtggcgggccataccaacaaactgaccaaaatgagcccggatgtgaccaaaagcaacatgaccgatgataaag
cgctgaactatgcggcgcagcaggcggcgagcctgggcagccagctgcaAagccgcagcctgaacggcgattatgcgaaagataccgcgctgggc
attgcgggcaaccaggcgagcagccagctgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttgatgg
cagcagcctggattttctgctgccgttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgctttaccgcgaacctg
ggcgcgggccagcgcttttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctgggcattggc

ICB

tcaggattttagcggcgataacacccgcctgggcattggcggcgaatattggcgcgattattttaaaagcagcgtgaacggctattttcgcatgagcggctg
gcatgaaagctataacaaaaaagattatgatgaacgcccggcgaacggctttgatattcgctttaacggctatctgccgagctatccggcgctgggcgcg
aaactgatttatgaacagtattatggcgataacgtggcgctgtttaacagcgataaactgcaAagcaacccgggcgcggcgaccgtgggcgtgaactat
accccgattccgctggtgaccatgggcattgattatcgccatggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgctatcagtttgataaa
agctggagccagcagattgaaccgcagtatgtgaacgaactgcgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattct
ggaatataaaaaacaggatattctgagcctgaacattccgcatgatattaacggcaccgaacatagcacccagaaaattcagctgattgtgaaaagca
aatatggcctggatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcatagcggcagccagagcgcgcaggattatca
ggcgattctgccggcgtatgtgcagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcgcaacggcaacagcagcaacaacgtgca
gctgaccattaccgtgctgagcaacggccaggtggtggatcaggtgggcgtgaccgattttaccgcggataaaaccagcgcgaaagcggataacgcg
gataccattacctataccgcgaccgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgagcttaacattgtgagcggcaccgcgaccctgg
gcgcgaacagc

ICC

tttaacattgtgagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccctgaaaagcagcaccc
cgggccaggtggtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgagcggca
gcggcggcagcggcatggcgggcgtggataccctgagcggcctgagcagcgaacagggccagagcggcgatatgaccattgaagaagatagcgc
gacccatattaaatttagcaaacgcgatgaagatggcaaagaactggcgggcgcgaccatggaactgcgcgatagcagcggcaaaaccattagcac

ctggattagcgatggccaggtgaaagatttttatctgtatccgggcaaatatacctttgtggaaaccgcggcgccggatggctatgaagtggcgaccgcg
attacctttaccgtgaacgaacagggccaggtgaccgtgaacggcaaagcgaccaaaggcgatgcgcatattaccggctactagagccaggcatca
aataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggc
ctttctgcgtttatatactagtagcggccgctgcag

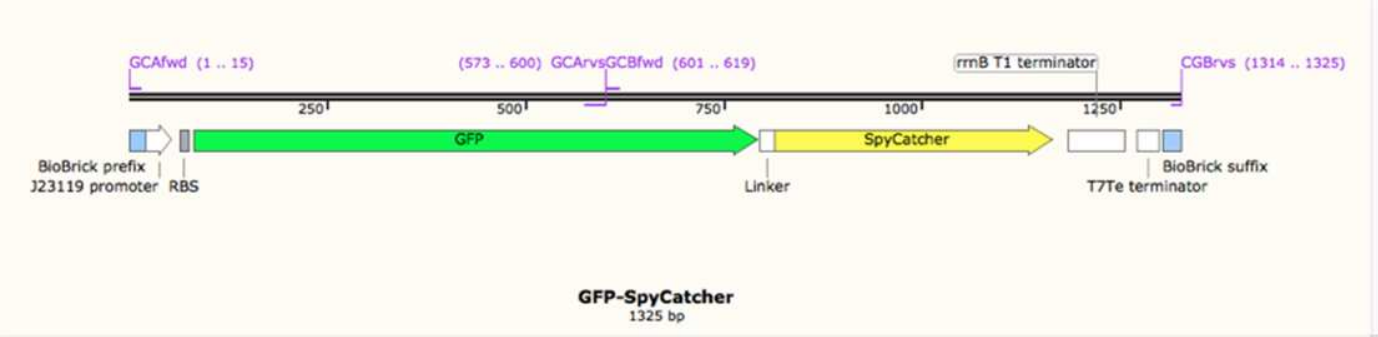
4. GFP SpyTag

3. GFPSpy1: PROM-RBS-GFP-SpyTag-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgcgtaaaggagaagaacttttcactgg
agttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgca
ctactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgagatacccagatcatatgaaacagcatgactttttcaagagtgc
catgcccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactacaagacacgtgctgaagtcaagtttgaaggtgataccttgttaatagaatcga
gttaaaaggtattgattttaaagaagatggaaacattcttggacacaaattggaatacaactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaa
tcaaagttaacttcaaaattagacacaacattgaagatggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttaccagaca
accattacctgtccacacaatctgccctttcgaaagatcccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatg
aactatacaaaggcagcggcggcagcggcgcgcatattgtgatggtggatgcgtataaaccgaccaaatactagagtactagagccaggcatcaaataaaacgaaa
ggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacacttactagtagcggccgctgcag

Prefix/Suffix
GFP
Linker
SpyTag

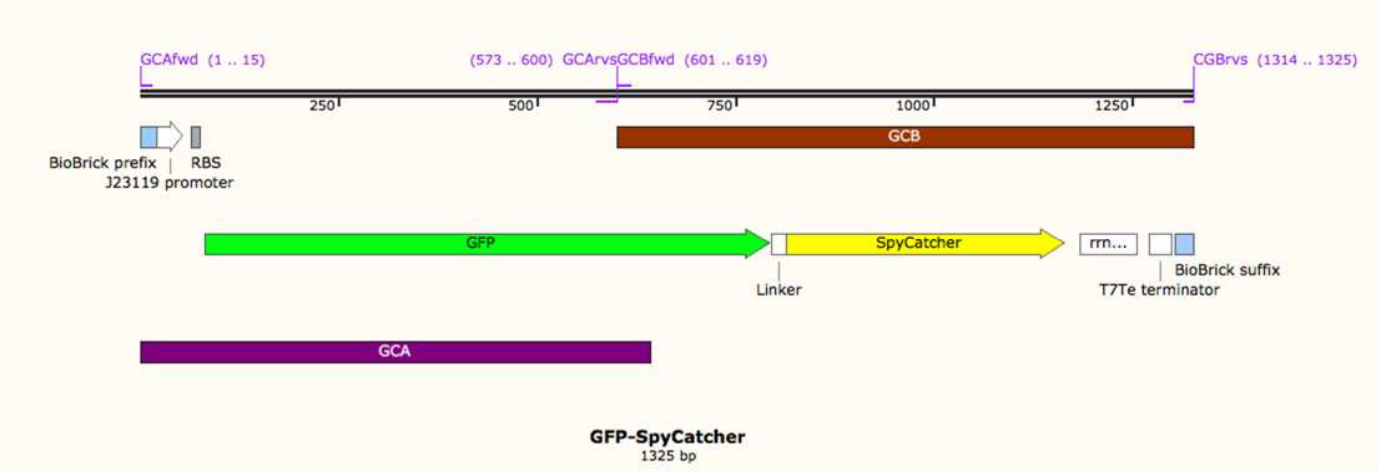
5. GFP SpyCatcher



GFPSpy2: PROM-RBS-GFP-SpyCatcher-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgcgtaaaggagaagaacttttcactgg
agttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgca
ctactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgagatacccagatcatatgaaacagcatgactttttcaagagtgc
catgcccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactacaagacacgtgctgaagtcaagtttgaaggtgataccttgttaatagaatcga
gttaaaaggtattgattttaaagaagatggaaacattcttggacacaaattggaatacaactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaa
tcaaagttaacttcaaaattagacacaacattgaagatggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttaccagaca
accattacctgtccacacaatctgccctttcgaaagatcccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatg
aactatacaaaggcagcggcggcagcggcatggcgggcgtggataccctgagcggcctgagcagcgaacagggccagagcggcgatatgaccattgaagaagat
agcgcgacccatattaaatttagcaaacgcgatgaagatggcaaagaactggcgggcgcgaccatggaactgcgcgatagcagcggcaaaaccattagcacctgg
attagcgatggccaggtgaaagatttttatctgtatccgggcaaatatacctttgtggaaaccgcggcgccggatggctatgaagtggcgaccgcgattacctttaccgtga
acgaacagggccaggtgaccgtgaacggcaaagcgaccaaaggcgatgcgcatattaccggctactagagccaggcatcaaataaaacgaaaggctcagtcgaa
agactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
Intimin’
Linker
SpyCatcher

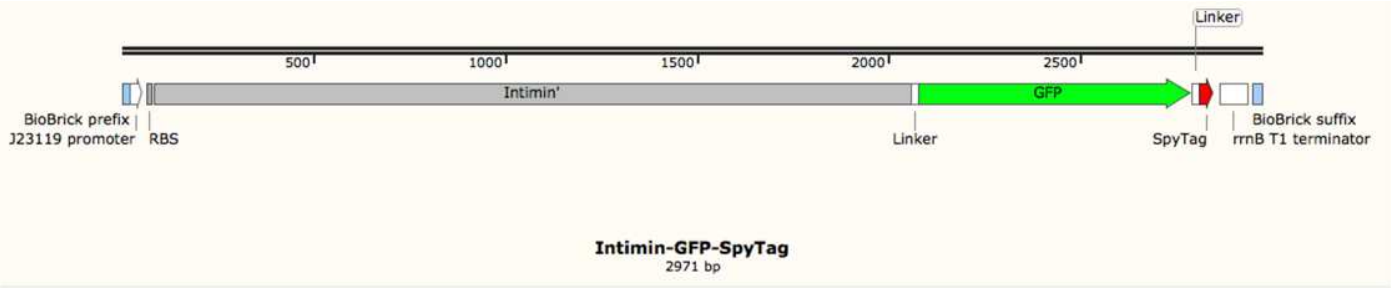


GCA
gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgcgtaaaggagaaga
acttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaa
aacttacccttaaatttatttgcactactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgagatacccagat
catatgaaacagcatgacttttcaagagtgccatgcccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactacaagacacgtg
ctgaagtcaagtttgaaggtgataccttgttaatagaatcgagttaaaaggtattgattttaaagaagatggaaacattcttggacacaaattggaataca
actataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattagacacaacattgaagatggaagcgttc
aactagcagaccattatcaacaaaatactcca

GCB
ggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtcttttaccagacaaccattacctgtccacacaatctgcccttt
cgaaagatcccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaactatacaaaggcagc
ggcggcagcggcatggcgggcgtggataccctgagcggcctgagcagcgaacagggccagagcggcgatatgaccattgaagaagatagcgcga

cccatattaaatttagcaaacgcgatgaagatggcaaagaactggcgggcgcgaccatggaactgcgcgatagcagcggcaaaaccattagcacct
ggattagcgatggccaggtgaaagatttttatctgtatccgggcaaatatacctttgtggaaaccgcggcgccggatggctatgaagtggcgaccgcgatt
acctttaccgtgaacgaacagggccaggtgaccgtgaacggcaaagcgaccaaaggcgatgcgcatattaccggctactagagccaggcatcaaat
aaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttt
ctgcgtttatatactagtagcggccgctgcag

1. Intimin GFP SpyTag

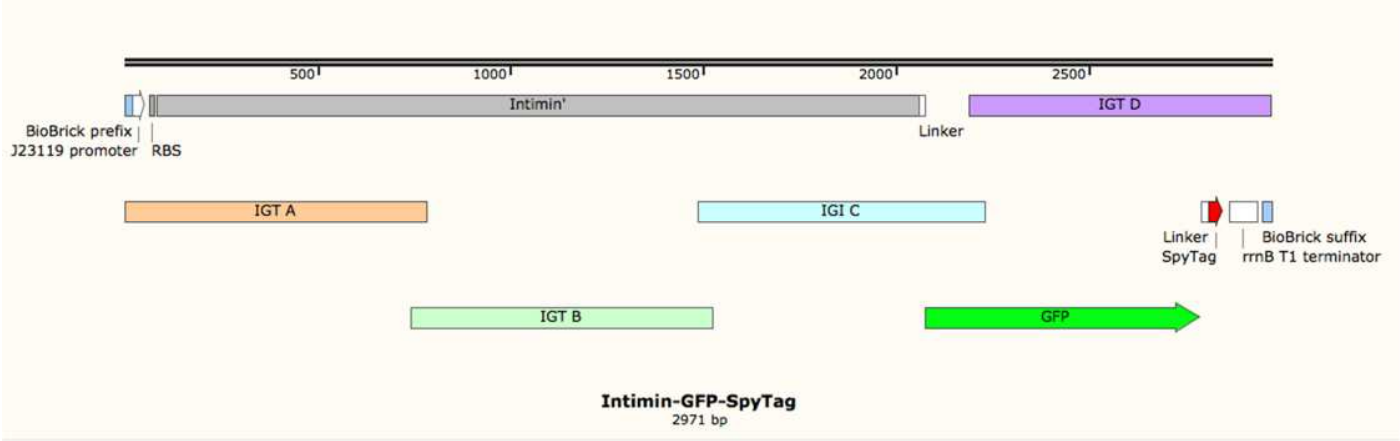


IGT: PROM-RBS-Intimin'-GFP-SpyTag-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgattacccatggctgctatacccgcacccg
ccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagctttgcgaacggcgaaaactattttaaactgggcag
cgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggcgaaaccgtggcggatctgagcaaaagccaggatattaacctgagcac
catttggagcctgaacaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgccgggccagcagattattctgccgctgaaaaaactgccgtttgaatatagcgc
gctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtggcgggccataccaacaaactgaccaaaatgagcccggatgtgaccaaaagcaacatgaccg
atgataaagcgctgaactatgcggcgcagcaggcggcgagcctgggcagccagctgcaAagccgcagcctgaacggcgattatgcgaaagataccgcgctgggcattg
cgggcaaccaggcgagcagccagctgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttgatggcagcagcctggattttct
gctgccgttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgctttaccgcgaacctgggcgcgggccagcgctttttctgccggcg
aacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctgggcattggcggcgaatattggcgcgattattttaaaagcagcgtgaacggctatttt
cgcatgagcggctggcatgaaagctataacaaaaaagattatgatgaacgcccggcgaacggctttgatattcgctttaacggctatctgccgagctatccggcgctgggcg
cgaaactgatttatgaacagtattatggcgataacgtggcgctgtttaacagcgataaactgcaAagcaacccgggcgcggcgaccgtgggcgtgaactataccccgattcc
gctggtgaccatgggcattgattatcgccatggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgctatcagtttgataaaagctggagccagcagattgaacc
gcagtatgtgaacgaactgcgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattctggaatataaaaaacaggatattctgagcctgaacatt
ccgcatgatattaacggcaccgaacatagcacccagaaaattcagctgattgtgaaaagcaaatatggcctggatcgcattgtgtgggatgatagcgcgctgcgcagccag
ggcggccagattcagcatagcggcagccagagcgcgcaggattatcaggcgattctgccggcgtatgtgcagggcggcagcaacatttataaagtgaccgcgcgcgcgt
atgatcgcaacggcaacagcagcaacaacgtgcagctgaccattaccgtgctgagcaacggccaggtggtggatcaggtgggcgtgaccgattttaccgcggataaaac
cagcgcgaaagcggataacgcggataccattacctataccgcgaccgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgagctttaacattgtgagcggcac
cgcgaccctgggcgcgaacagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccctgaaaagcagcaccccgggccaggtggtggtgagcgcgaaaaccg
cggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgagcggcagcggcggcagcggcAtgcgtaaaggagaagaacttttcactgg
agttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcacta
ctggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgagatacccagatcatatgaaacagcatgacttttcaagagtgccatgcc
cgaaggttatgtacaggaaagaactatattttcaaagatgacgggaactacaagacacgtgctgaagtcaagtttgaaggtgataccttgttaatagaatcgagttaaaag
gtattgattttaaagaagatggaaacattcttggacacaaattggaatacaactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaac
ttcaaaattagacacaacattgaagatggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttaccagacaaccattacctgtcc
acacaatctgccctttcgaaagatcccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaactatacaaaggcag
cggcggcagcggcgcgcatattgtgatggtggatgcgtataaaccgaccaaatactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgt
tttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
Promoter
Ribosomal binding site
Intimin'
GFP
Linker
SpyTag
Terminator

Assembly through Gibson or in-fusion assembly



IGTA

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgattacccatggctgctatac
ccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagctttgcgaacggcga
aaactattttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggcgaaaccgtggcggatctg
agcaaaagccaggatattaacctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgccgggccagca
gattattctgccgctgaaaaaactgccgtttgaatatagcgcgctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtggcgggccatacca
acaaactgaccaaaatgagcccggatgtgaccaaaagcaacatgaccgatgataaagcgctgaactatgcggcgcagcaggcggcgagcctgggcag
ccagctgcaAagccgcagcctgaacggcgattatgcgaaagataccgcgctgggcattgcgggcaaccaggcgagcagccagctgcaAgcgtggctg
caAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttgatggcagcagcctggattttctgctgccgttt

IGTB

aacaactttgatggcagcagcctggattttctgctgccgtttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgcttta
ccgcgaacctgggcgcgggccagcgctttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctgggc
attggcggcgaatattggcgcgattattttaaaagcagcgtgaacggctattttcgcatgagcggctggcatgaaagctataacaaaaaagattatgatgaac
gcccggcgaacggctttgatattcgctttaacggctatctgccgagctatccggcgctgggcgcgaaactgatttatgaacagtattatggcgataacgtggcg
ctgtttaacagcgataaactgcaAagcaacccgggcgcggcgaccgtgggcgtgaactataccccgattccgctggtgaccatgggcattgattatcgccat
ggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgctatcagtttgataaaagctggagccagcagattgaaccgcagtatgtgaacgaactg
cgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattctggaatataaaaaacaggatattctgagcctgaacattccgcatgat
attaacggcaccgaacatagcacccagaaaattcagctgattgtgaaaagcaaatatggc

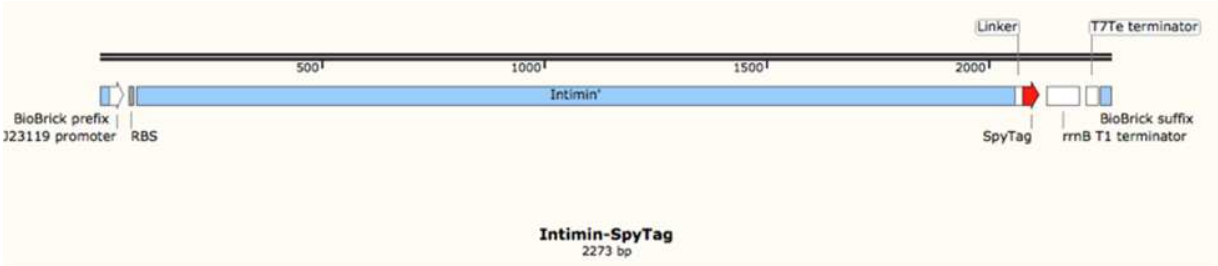
IGTC

cacccagaaaattcagctgattgtgaaaagcaaatatggcctggatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcata
gcggcagccagagcgcgcaggattatcaggcgattctgccggcgtatgtgcagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcgcaa
cggcaacagcagcaacaacgtgcagctgaccattaccgtgctgagcaacggccaggtggtggatcaggtgggcgtgaccgattttaccgcggataaaac
cagcgcgaaagcggataacgcggataccattacctataccgcgaccgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgagctttaacattgt
gagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccctgaaaagcagcaccccgggccaggtg
gtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgagcggcagcggcggcagcgg
cAtgcgtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggt
gatgcaacatacggaaaacttacccttaaatttatttgcactactgga

IGTD

catacggaaaacttacccttaaatttatttgcactactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgagata
cccagatcatatgaaacagcatgacttttttcaagagtgccatgcccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactacaagaca
cgtgctgaagtcaagtttgaaggtgataccttgttaatagaatcgagttaaaaggtattgattttaaagaagatggaaacattcttggacacaaattggaatac
aactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattagacacaacattgaagatggaagcgttca
actagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttaccagacaaccattacctgtccacacaatctgccctttcgaaagatccca
acgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaactatacaaaggcagcggcggcagcggcgc
gcatattgtgatggtggatgcgtataaaccgaccaaatactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgt
tgtttgtcggtgaacgctctctactagagtcacacttactagtagcggccgctgca

2. Intimin SpyTag

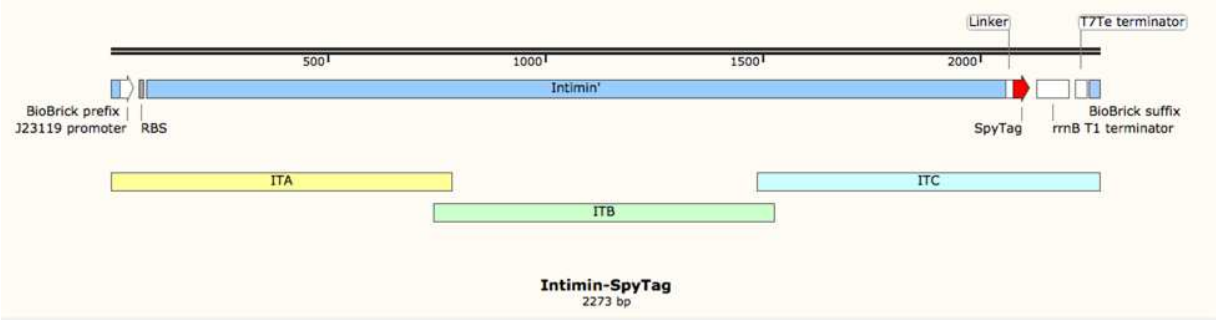


IT: PROM-RBS-Intimin'-SpyTag-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgattacccatggct
gctatacccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagcttt
gcgaacggcgaaaactattttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggc
gaaaccgtggcggatctgagcaaaagccaggatattaacctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcgaaatga
tgaaagcggcgccgggccagcagattattctgccgctgaaaaaactgccgtttgaatatagcgcgctgccgctgctgggcagcgcgccgctggtg
gcggcgggcggcgtggcgggccataccaacaaactgaccaaaatgagcccggatgtgaccaaaagcaacatgaccgatgataaagcgctga
actatgcggcgcagcaggcggcgagcctgggcagccagctgcaAagccgcagcctgaacggcgattatgcgaaagataccgcgctgggcatt
gcgggcaaccaggcgagcagccagctgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttgatgg
cagcagcctggattttctgctgccgttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgctttaccgcgaac
ctgggcgcgggccagcgctttttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctgggcattg
gcggcgaatattggcgcgattattttaaaagcagcgtgaacggctattttcgcatgagcggctggcatgaaagctataacaaaaaagattatgatga
acgcccggcgaacggctttgatattcgctttaacggctatctgccgagctatccggcgctgggcgcgaaactgatttatgaacagtattatggcgataa
cgtggcgctgtttaacagcgataaactgcaAagcaacccgggcgcggcgaccgtgggcgtgaactataccccgattccgctggtgaccatgggc
attgattatcgccatggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgctatcagtttgataaaagctggagccagcagattgaacc
gcagtatgtgaacgaactgcgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattctggaatataaaaaacaggata
ttctgagcctgaacattccgcatgatattaacggcaccgaacatagcacccagaaaattcagctgattgtgaaaagcaaatatggcctggatcgcat
tgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcatagcggcagccagagcgcgcaggattatcaggcgattctgccggcg
tatgtgcagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcgcaacggcaacagcagcaacaacgtgcagctgaccattaccg
tgctgagcaacggccaggtggtggatcaggtgggcgtgaccgattttaccgcggataaaaccagcgcgaaagcggataacgcggataccattac
ctataccgcgaccgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgagctttaacattgtgagcggcaccgcgaccctgggcgcgaa
cagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccctgaaaagcagcaccccgggccaggtggtggtgagcgcgaaaaccgcgg
aaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgagcggcagcggcggcagcggcgcgcatattgtgatggtg
gatgcgtataaaccgaccaaatactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggt
gaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Promoter
Ribosomal binding site
Prefix/Suffix
Intimin'
Linker
SpyTag
Terminator

Assembly through Gibson or in-fusion assembly



ITA

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgattac
ccatggctgctatacccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgt
gaaccagaacagctttgcgaacggcgaaaactattttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaacc
gcctgttttataccctgaaaaccggcgaaaccgtggcggatctgagcaaaagccaggatattaacctgagcaccatttggagcctga
acaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgccgggccagcagattattctgccgctgaaaaaactgccgttt
gaatatagcgcgctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtggcgggccataccaacaaactgaccaaa
atgagcccggatgtgaccaaaagcaacatgaccgatgataaagcgctgaactatgcggcgcagcaggcggcgagcctgggcag
ccagctgcaAagccgcagcctgaacggcgattatgcgaaagataccgcgctgggcattgcgggcaaccaggcgagcagccagc
tgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttgatggcagcagcctggattttctgc
tgccgttt

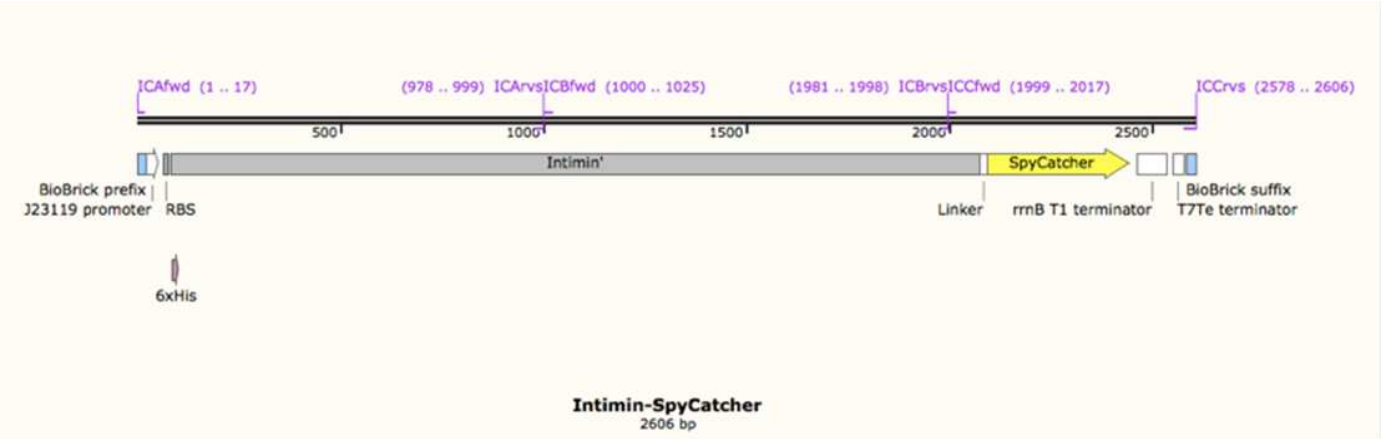
ITB

acaactttgatggcagcagcctggattttctgctgccgtttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatat
tgatagccgctttaccgcgaacctgggcgcgggccagcgctttttttctgccggcgaacatgctgggctataacgtgtttattgatcaggat
tttagcggcgataacacccgcctgggcattggcggcgaatattggcgcgattattttaaaagcagcgtgaacggctattttcgcatgagc
ggctggcatgaaagctataacaaaaaagattatgatgaacgcccggcgaacggctttgatattcgctttaacggctatctgccgagct
atccggcgctgggcgcgaaactgatttatgaacagtattatggcgataacgtggcgctgtttaacagcgataaactgcaAagcaacc
cgggcgcggcgaccgtgggcgtgaactataccccgattccgctggtgaccatgggcattgattatcgccatggcaccggcaacgaa
aacgatctgctgtatagcatgcagtttcgctatcagtttgataaaagctggagccagcagattgaaccgcagtatgtgaacgaactgcg
caccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattctggaatataaaaaacaggatattctgagcctgaa
cattccgcatgatattaacggcaccgaacatagcaccagaaaattcagctgattgtgaaaagcaaatatggcctg

ITC

ccagaaaattcagctgattgtgaaaagcaaatatggcctggatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggcc
agattcagcatagcggcagccagagcgcgcaggattatcaggcgattctgccggcgtatgtgcagggcggcagcaacatttataaa
gtgaccgcgcgcgcgtatgatcgcaacggcaacagcagcaacaacgtgcagctgaccattaccgtgctgagcaacggccaggtg
gtggatcaggtgggcgtgaccgattttaccgcggataaaaccagcgcgaaagcggataacgcggataccattacctataccgcgac
cgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgagctttaacattgtgagcggcaccgcgaccctgggcgcgaacag
cgcgaaaaccgatgcgaacggcaaagcgaccgtgaccctgaaaagcagcaccccgggccaggtggtggtgagcgcgaaaacc
gcggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgagcggcagcggcggcagcggcgcg
catattgtgatggtggatgcgtataaaccgaccaaatactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactggg
cctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagc
ggccgctgcag

3. Intimin SpyCatcher

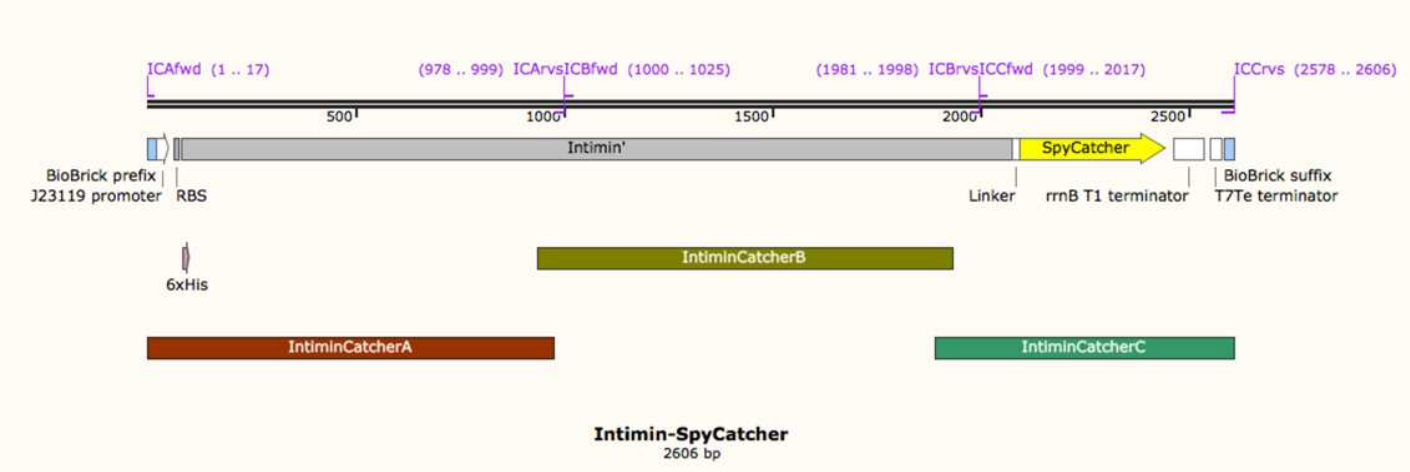


IC: PROM-RBS-V5tag-Intimin'-SpyCatcher-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgCACCATCACCATCACCATatt
acccatggctgctatacccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagctttgcgaa
cggcgaaaactatttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggcgaaaccgtggcggatctgag
caaaagccaggatattaacctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgccgggccagcagattattctgccg
ctgaaaaaactgccgtttgaatatagcgcgctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtggcgggccataccaacaaactgaccaaaatgagc
ccggatgtgaccaaaagcaacatgaccgatgataaagcgctgaactatgcggcgcagcaggcggcgagcctgggcagccagctgcaAagccgcagcctgaacggc
gattatgcgaaagataccgcgctgggcattgcgggcaaccaggcgagcagccagctgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcg
gcaacaactttgatggcagcagcctggattttctgctgccgttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgctttaccgcgaa
cctgggcgcgggccagcgctttttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctgggcattggcggcgaatattg
gcgcgattattttaaaagcagcgtgaacggctattttcgcatgagcggctggcatgaaagctataacaaaaaagattatgatgaacgcccggcgaacggctttgatattcgct
ttaacggctatctgccgagctatccggcgctgggcgcgaaactgatttatgaacagtattatggcgataacgtggcgctgtttaacagcgataaactgcaAagcaacccggg
cgcggcgaccgtgggcgtgaactataccccgattccgctggtgaccatgggcattgattatcgccatggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgc
tatcagtttgataaaagctggagccagcagattgaaccgcagtatgtgaacgaactgcgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattatt
ctggaatataaaaaacaggatattctgagcctgaacattccgcatgatattaacggcaccgaacatagcacccagaaaattcagctgattgtgaaaagcaaatatggcctg
gatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcatagcggcagccagagcgcgcaggattatcaggcgattctgccggcgtatgtgc
agggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcgcaacggcaacagcagcaacaacgtgcagctgaccattaccgtgctgagcaacggccaggtg
gtggatcaggtgggcgtgaccgattttaccgcggataaaaccagcgcgaaagcggataacgcggataccattacctataccgcgaccgtgaaaaaaaacggcgtggcg
caggcgaacgtgccggtgagctttaacattgtgagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccctgaaaa
gcagcaccccgggccaggtggtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgagcggcagc
ggcggcagcggcatggcgggcgtggataccctgagcggcctgagcagcgaacagggccagagcggcgatatgaccattgaagaagatagcgcgacccatattaaatt
tagcaaacgcgatgaagatggcaaagaactggcgggcgcgaccatggaactgcgcgatagcagcggcaaaaccattagcacctggattagcgatggccaggtgaaa
gatttttatctgtatccgggcaaatatacctttgtggaaaccgcggcgccggatggctatgaagtggcgaccgcgattacctttaccgtgaacgaacagggccaggtgaccgt
gaacggcaaagcgaccaaaggcgatgcgcatattaccggctactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgttt
gtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
Promoter
Ribosomal binding site
Intimin'
Linker
SpyCatcher
6xHis
Terminator

Assembly through Gibson or in-fusion assembly



IntiminCatcherA (ICA)

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgCACCATCACCAT
CACCATattacccatggctgctatacccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtg
aaccagaacagctttgcgaacggcgaaaactattttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctg
aaaaccggcgaaaccgtggcggatctgagcaaaagccaggatattaacctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcga
aatgatgaaagcggcgccgggccagcagattattctgccgctgaaaaaactgccgtttgaatatagcgcgctgccgctgctgggcagcgcgccgctggtg
gcggcgggcggcgtggcgggccataccaacaaactgaccaaaatgagcccggatgtgaccaaaagcaacatgaccgatgataaagcgctgaactat
gcggcgcagcaggcggcgagcctgggcagccagctgcaAagccgcagcctgaacggcgattatgcgaaagataccgcgctgggcattgcgggcaac
caggcgagcagccagctgcaAgcgtggctgcaAcattatggcaccgcggaagtgaacctgcaAagcggcaacaactttgatggcagcagcctggattt
tctgctgccgttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgatagccgctttaccgcgaacctgggcgcgggccagcgc
ttttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctgggcattggc

ICB

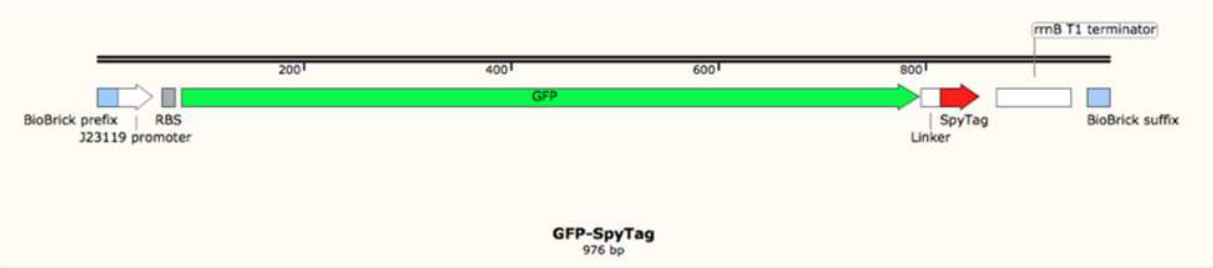
tcaggattttagcggcgataacacccgcctgggcattggcggcgaatattggcgcgattattttaaaagcagcgtgaacggctattttcgcatgagcggctgg
catgaaagctataacaaaaaagattatgatgaacgcccggcgaacggctttgatattcgctttaacggctatctgccgagctatccggcgctgggcgcgaa
actgatttatgaacagtattatggcgataacgtggcgctgtttaacagcgataaactgcaAagcaacccgggcgcggcgaccgtgggcgtgaactataccc
cgattccgctggtgaccatgggcattgattatcgccatggcaccggcaacgaaaacgatctgctgtatagcatgcagtttcgctatcagtttgataaaagctgg
agccagcagattgaaccgcagtatgtgaacgaactgcgcaccctgagcggcagccgctatgatctggtgcagcgcaacaacaacattattctggaatata
aaaaacaggatattctgagcctgaacattccgcatgatattaacggcaccgaacatagcacccagaaaattcagctgattgtgaaaagcaaatatggcct
ggatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcatagcggcagccagagcgcgcaggattatcaggcgattctgcc
ggcgtatgtgcagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcgcaacggcaacagcagcaacaacgtgcagctgaccattaccgt
gctgagcaacggccaggtggtggatcaggtgggcgtgaccgattttaccgcggataaaaccagcgcgaaagcggataacgcggataccattacctatac
cgcgaccgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgagcttaacattgtgagcggcaccgcgaccctgggcgcgaacagc

ICC

tttaacattgtgagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccgatgcgaacggcaaagcgaccgtgaccctgaaaagcagcaccccg
ggccaggtggtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgcggtgatttttttgatcagaccaaagcgagcggcagcgg

cggcagcggcatggcgggcgtggataccctgagcggcctgagcagcgaacagggccagagcggcgatatgaccattgaagaagatagcgcgaccc
atattaaatttagcaaacgcgatgaagatggcaaagaactggcgggcgcgaccatggaactgcgcgatagcagcggcaaaaccattagcacctggatt
agcgatggccaggtgaaagatttttatctgtatccgggcaaatataccttgtggaaaccgcggcgccggatggctatgaagtggcgaccgcgattaccttta
ccgtgaacgaacagggccaggtgaccgtgaacggcaaagcgaccaaaggcgatgcgcatattaccggctactagagccaggcatcaaataaaacga
aaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttata
tactagtagcggccgctgcag

4. GFP SpyTag

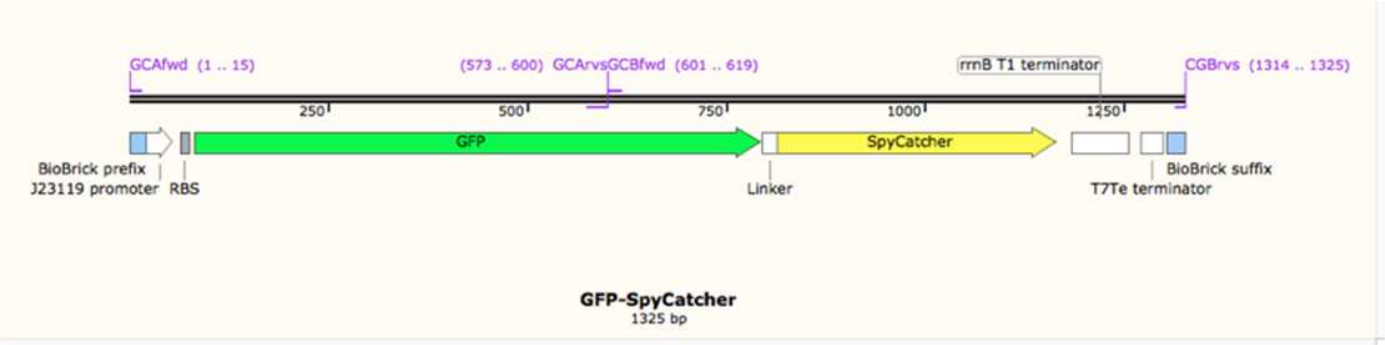


3. GFPSpy1: PROM-RBS-GFP-SpyTag-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgcgtaaaggagaa
gaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatac
ggaaaacttacccttaaatttatttgcactactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgagat
acccagatcatatgaaacagcatgactttttcaagagtgccatgcccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactac
aagacacgtgctgaagtcaagtttgaaggtgataccttgttaatagaatcgagttaaaaggtattgattttaaagaagatggaaacattcttggacac
aaattggaatacaactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattagacacaacatt
gaagatggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttaccagacaaccattacctgtccacac
aatctgccctttcgaaagatcccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaacta
tacaaaggcagcggcggcagcggcgcgcatattgtgatggtggatgcgtataaaccgaccaaatactagagtactagagccaggcatcaaataa
aacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacacttactagtagcggccgctgcag

Prefix/Suffix
Promoter
Ribosomal binding site
GFP
Linker
SpyTag
Terminator

5. GFP SpyCatcher

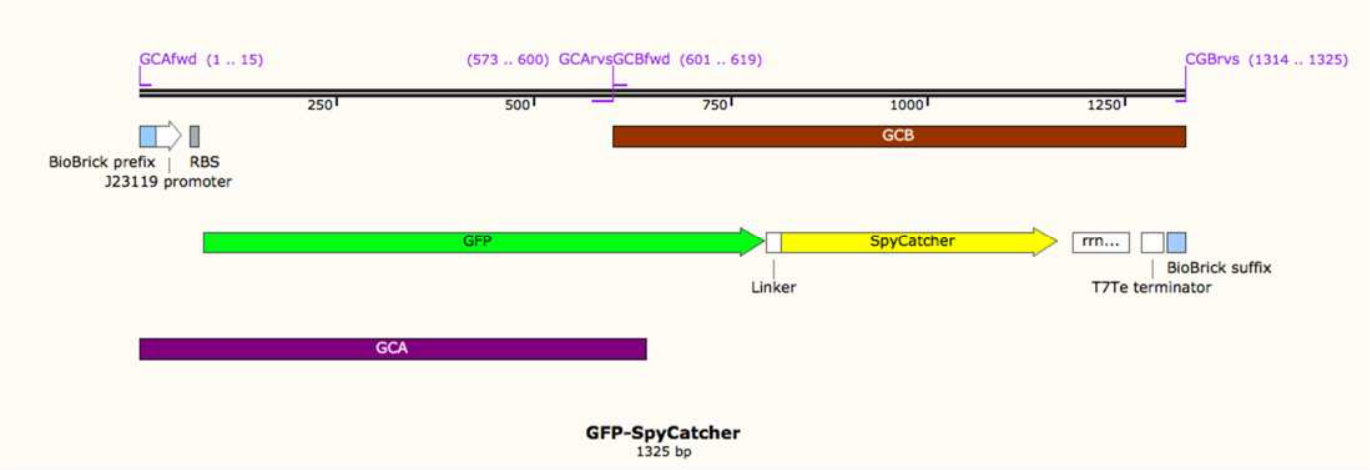


GFPSpy2: PROM-RBS-GFP-SpyCatcher-TT

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgcgtaaaggagaagaacttttcactgga
gttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcacta
ctggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgagatacccagatcatatgaaacagcatgacttttcaagagtgccatgc
ccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactacaagacacgtgctgaagtcaagtttgaaggtgataccttgttaatagaatcgagttaaaa
ggtattgattttaaagaagatggaaacattcttggacacaaattggaatacaactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagtta
acttcaaaattagacacaacattgaagatggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttaccagacaaccattacctgt
ccacacaatctgccctttcgaaagatcccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaactatacaaagg
cagcggcggcagcggcatggcgggcgtggataccctgagcggcctgagcagcgaacagggccagagcggcgatatgaccattgaagaagatagcgcgacccatatt
aaatttagcaaacgcgatgaagatggcaaagaactggcgggcgcgaccatggaactgcgcgatagcagcggcaaaaccattagcacctggattagcgatggccaggt
gaaagatttttatctgtatccgggcaaatatacctttgtggaaaccgcggcgccggatggctatgaagtggcgaccgcgattacctttaccgtgaacgaacagggccaggtg
accgtgaacggcaaagcgaccaaaggcgatgcgcatattaccggctactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatct
gttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
Intimin’
Linker
SpyCatcher

Assembly through Gibson or in-fusion assembly



GCA

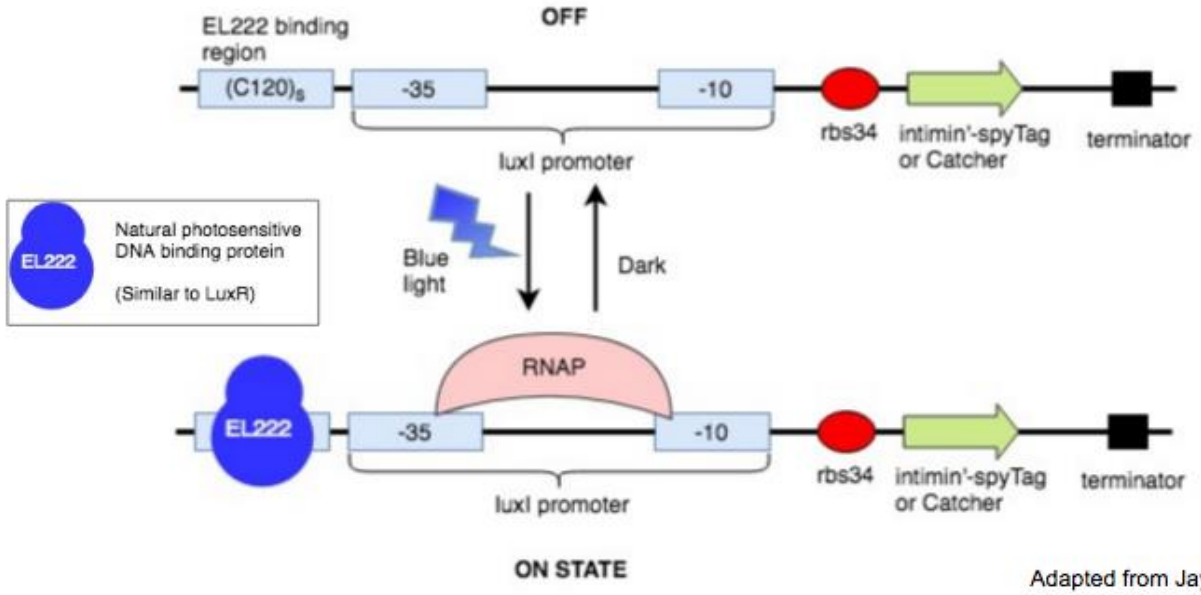
gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgcgtaaaggagaagaac
ttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaaaactt
acccttaaatttatttgcactactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgagatacccagatcatatg
aaacagcatgactttttcaagagtgccatgcccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactacaagacacgtgctgaagtc
aagtttgaaggtgataccttgttaatagaatcgagttaaaaggtattgattttaaagaagatggaaacattcttggacacaaattggaatacaactataactc
acacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattagacacaacattgaagatggaagcgttcaactagcaga
ccattatcaacaaaatactcca

GCB

ggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtcctttaccagacaaccattacctgtccacacaatctgccctttc
gaaagatcccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaactatacaaaggcagcggc
ggcagcggcatggcgggcgtggataccctgagcggcctgagcagcgaacagggccagagcggcgatatgaccattgaagaagatagcgcgacccat
attaaatttagcaaacgcgatgaagatggcaaagaactggcgggcgcgaccatggaactgcgcgatagcagcggcaaaaccattagcacctggattag
cgatggccaggtgaaagatttttatctgtatccgggcaaatatacctttgtggaaaccgcggcgccggatggctatgaagtggcgaccgcgattacctttacc
gtgaacgaacagggccaggtgaccgtgaacggcaaagcgaccaaaggcgatgcgcatattaccggctactagagccaggcatcaaataaaacgaaa
ggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatac
tagtagcggccgctgcag

PART B: Light induction

1. **Blue-light inducible promoter (Transcriptional control)**



Adapted from Jayaraman et al., 2016

1. EL222 binding region and promoter: PBlind-v1-rbs34-GFP (962bp)

gaattcgcggccgcttctagGGTAGCCTTTAGTCCATGttagcgaagaaaatggtttgtTATAGTcgaataaaAAAGAGGAGAAAtatacatAtgcgtaaaggagaa
gaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgc
actactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgctttgcgagatacccagatcatatgaaacagcatgactttttcaagagtgccatgcccgaag
gttatgtacaggaaagaactatatttttcaaagatgacgggaactacaagacacgtgctgaagtcaagtttgaaggtgataccttgttaatagaatcgagttaaaaggtattgattttaaagaa
gatggaaacattcttggacacaaattggaatacaactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattagacacaacattgaag
atggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttaccagacaaccattacctgtccacacaatctgccctttcgaaagatcccaacgaaa
agagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaactatacaaataataaccaggcatcaaataaaacgaaaggctcagtcgaaagactgg
gcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
EL222 binding region, -35 hexamer, -10 hexamer
Rbs34
GFP ← **WILL BE REPLACED BY OUR FUSION PROTEINS**
Double terminator

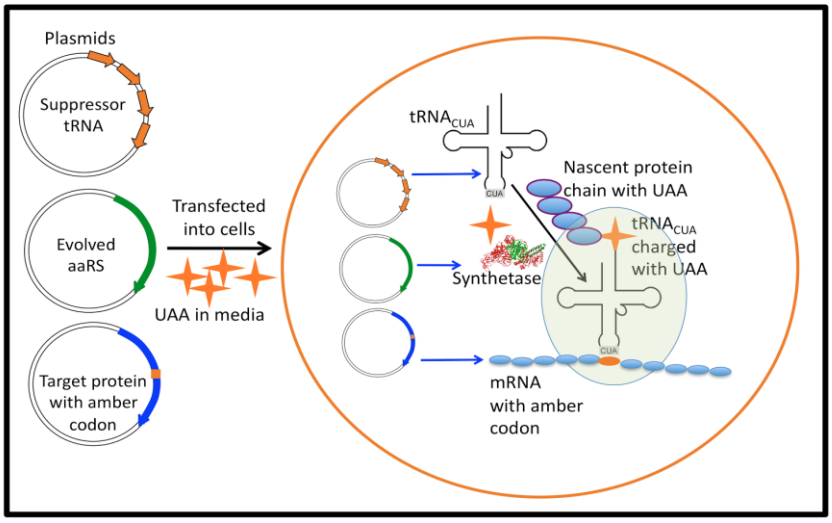
2. EL222: rrnBp1-rbs34-EL222 (constitutive promoter) (906bp)

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagatgctggatatgggccaggatcgcccgattgatggcagcgg
cgcgccgggcgcggatgatacccgcgtggaagtgcagccgccggcgcagtgggtgctggatctgattgaagcgagcccgattgcgagcgtggtgagcgatccgcgcctggcggataacc
ccgctgattgcgattaaccaggcgtttaccgatctgaccggctatagcgaagaagaatgcgtgggccgcaactgccgctttctggcgggcagcggcaccgaaccgtggctgaccgataaa
attcgccagggcgtgcgcgaacataaaccggtgctggtggaaattctgaactataaaaaagatggcaccccgtttcgcaacgcggtgctggtggcgccgatttatgatgatgatgatgaact
gctgtattttctgggcagccaggtggaagtggatgatgatcagccgaacatgggcatggcgcgccgcgaacgcgcggcggaaatgctgaaaaccctgagcccgcgccagctggaagtg
accaccctggtggcgagcggcctgcgcaacaaagaagtggcggcgcgcctgggcctgagcgaaaaaaccgtgaaaatgcatcgcggcctggtgatggaaaaactgaacctgaaaac
cagcgcggatctggtgcgcattgcggtggaagcgggcattccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctacta
gagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
Constitutive Promoter
RBS
EL222
Double terminator

PART B: Light induction

2. Photocageing - Post-translational control



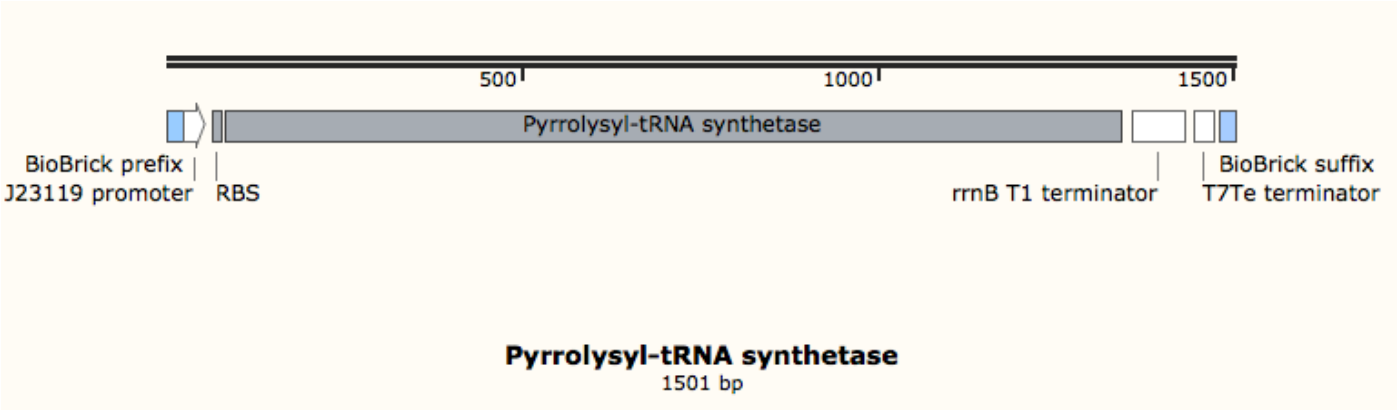
1. tRNA-Pyl (pylT)
2. Pyrrolysyl-tRNA synthetase
3. *Nε*-methyl-L-lysine
4. Protein with amber codon

Charging of $tRNA_{CUA}$ with pyrrolysine

Incorporating pyrrolysine onto the amber codon

Photocaging lysine residue on SpyCatcher

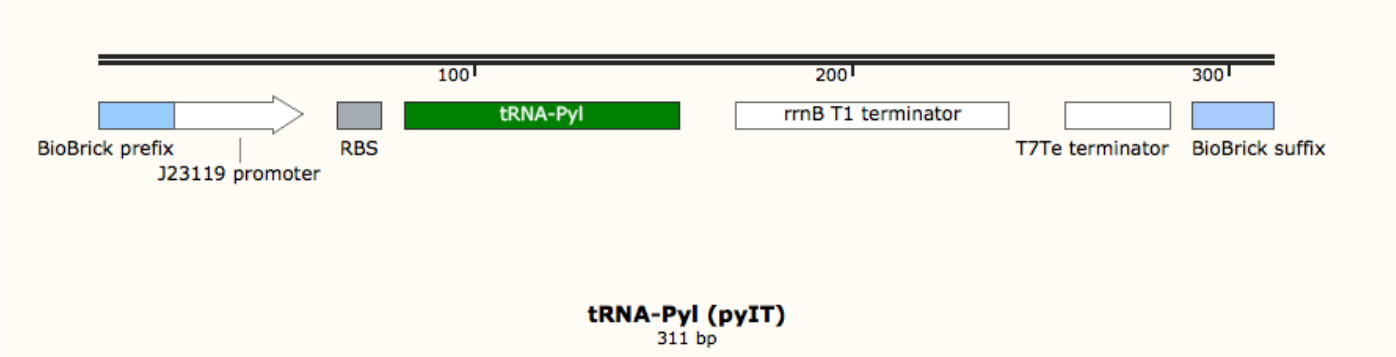
To verify that this peak represents photocaged methyl lysine, protein was subjected to photolysis for 20 minutes with ≥365 nm light



Pyrrolysyl-tRNA synthetase CDS (1501bp)
http://parts.igem.org/Part:BBa_K1223013
gaattcgcggccgcttctagagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagatggataaaaaaccattagatg
ttttaatatctgcgaccgggctctggatgtccaggactggcacgctccacaaaatcaaacactatgaggtctcaagaagtaaaatatacattgaaatggcg
tgtggagaccatcttgttgtgaataattctaggagttgtagaacagccagagcattcagacatcataagtacagaaaaacctgcaaacgatgtagggtttc
ggacgaggatatcaataatttcctcacaagatcaactgaaggcaaaaccagtgtgaaagttaaggtagtttctgctccaaaggtcaaaaaagctatgcc
gaaatcagtttcgagggctccaaagcctctggaaaatcctgtgtctgcaaaggcatcaacggacacatccagatctgtaccttcgcctgcaaaatcaact
ccaaattcgcctgttcccacatcggctcctgctccttcacttacaagaagccagctcgatagggttgaggctctcttaagtccagaggataaaatttctctga
atattgcaaagcctttcagggaacttgagtccgaacttgtgacaagaagaaaaaacgattttcagcggctctataccaatgatagagaagactaccttggt
aaactcgaacgggacattacgaaatttttcgtagaccgggattttctggagataaagtctcctatccttattccggcagaatacgtggagagaatgggtatta
acaatgatactgaactttcaaaacagatcttcagggtggataaaaatctctgcttaaggccaatgcttgccccgactctttacaactatctgcgaaaactcg
ataggattttaccagatcctataaagattttcgaagtcgggccctgttaccggaaagagtctgacggcaaagagcacctggaagaatttaccatggtgaa
cttctgtcagatgggttcgggatgtactcgggaaaatcttgaatccctcatcaaagagtttctggactatctggaaatcgacttcgaaatcgtaggagattcct
gtatggtctatggggataccttgatataatgcacggggacctggagctttcttcggcagtcgtcgggccagttcctcttgatagggaatggggcattgaca
aaccatggataggtgcaggttttgggcttgaacgcttgctcaaggttatgcatggctttaaaaacattaagagagcatcaaggtccgaatcttactataatgg

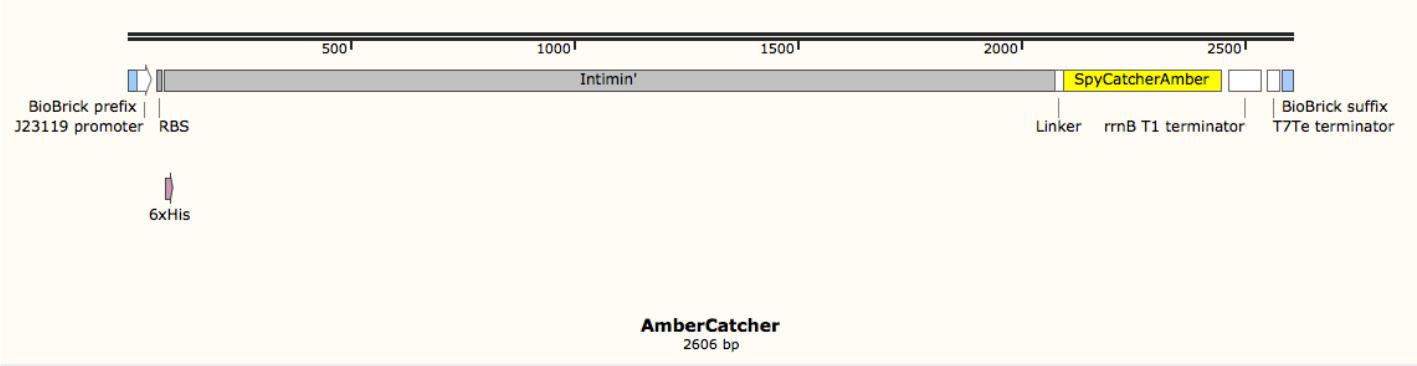
gatttcaaccaatctatgatagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
Pyrrolysyl-tRNA synthetase
Double terminator



tRNA-Pyl (pylT) gene (311bp)
http://parts.igem.org/Part:BBa_K1223014
gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagggaacctgatcatgtagatcgaatggactctaaatccgttcagccgggttagattcccgggtttccgccatagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
tRNA-Pyl
Double terminator



>PROM-RBS-antiV5-Intimin'-SpyCatcherAmber-TT (2606bp)

(k, aaa❼tag, stop)

gaattcgcggccgcttctagttgacagctagctcagtcctaggtataatgctagctactagagaaagaggagaaatactagAtgCACCATCACCATCACCAT
attacccatggctgctatacccgcacccgccataaacataaactgaaaaaaaccctgattatgctgagcgcgggcctgggcctgttttttatgtgaaccagaacagctttg
cgaacggcgaaaactattttaaactgggcagcgatagcaaactgctgacccatgatagctatcagaaccgcctgttttataccctgaaaaccggcgaaaccgtggcgg
atctgagcaaaagccaggatattaacctgagcaccatttggagcctgaacaaacatctgtatagcagcgaaagcgaaatgatgaaagcggcgccgggccagcagat
tattctgccgctgaaaaaactgccgtttgaatatagcgcgctgccgctgctgggcagcgcgccgctggtggcggcgggcggcgtggcgggccataccaacaaactgac
caaaatgagcccggatgtgaccaaaagcaacatgaccgatgataaagcgctgaactatgcggcgcagcaggcggcgagcctgggcagccagctgcaAagccgc
agcctgaacggcgattatgcgaaagataccgcgctgggcattgcgggcaaccaggcgagcagccagctgcaAgcgtggctgcaAcattatggcaccgcggaagtg
aacctgcaAagcggcaacaactttgatggcagcagcctggattttctgctgccgttttatgatagcgaaaaaatgctggcgtttggccaggtgggcgcgcgctatattgata
gccgctttaccgcgaacctgggcgcgggccagcgctttttctgccggcgaacatgctgggctataacgtgtttattgatcaggattttagcggcgataacacccgcctggg
cattggcggcgaatattggcgcgattattttaaaagcagcgtgaacggctattttcgcatgagcggctggcatgaaagctataacaaaaaagattatgatgaacgcccgg
cgaacggctttgatattcgctttaacggctatctgccgagctatccggcgctgggcgcgaaactgatttatgaacagtattatggcgataacgtggcgctgtttaacagcgat
aaactgcaAagcaacccgggcgcggcgaccgtgggcgtgaactataccccgattccgctggtgaccatgggcattgattatcgccatggcaccggcaacgaaaacg
atctgctgtatagcatgcagtttcgctatcagtttgataaaagctggagccagcagattgaaccgcagtatgtgaacgaactgcgcaccctgagcggcagccgctatgatc
tggtgcagcgcaacaacaacattattctggaatataaaaaacaggatattctgagcctgaacattccgcatgatattaacggcaccgaacatagcacccagaaaattca
gctgattgtgaaaagcaaatatggcctggatcgcattgtgtgggatgatagcgcgctgcgcagccagggcggccagattcagcatagcggcagccagagcgcgcagg
attatcaggcgattctgccggcgtatgtgcagggcggcagcaacatttataaagtgaccgcgcgcgcgtatgatcgcaacggcaacagcagcaacaacgtgcagctg
accattaccgtgctgagcaacggccaggtggtggatcaggtgggcgtgaccgattttaccgcggataaaaccagcgcgaaagcggataacgcggataccattacctat
accgcgaccgtgaaaaaaaacggcgtggcgcaggcgaacgtgccggtgagctttaacattgtgagcggcaccgcgaccctgggcgcgaacagcgcgaaaaccga
tgcgaacggcaaagcgaccgtgaccctgaaaagcagcaccccgggccaggtggtggtgagcgcgaaaaccgcggaaatgaccagcgcgctgaacgcgagcgc
ggtgatttttttgatcagaccaaagcgagcggcagcggcggcagcggcAtggcgggcgtggataccctgagcggcctgagcagcgaacagggccagagc
ggcgatatgaccattgaagaagatagcgcgacccatatttagtttagcaaacgcgatgaagatggcaaagaactggcgggcgcgaccatggaactgc
gcgatagcagcggcaaaaccattagcacctggattagcgatggccaggtgaaagatttttatctgtatccgggcaaatatacctttgtggaaaccgcggc
gccggatggctatgaagtggcgaccgcgattacctttaccgtgaacgaacagggccaggtgaccgtgaacggcaaagcgaccaaaggcgatgcgc
atattaccggctagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggct
caccttcgggtgggcctttctgcgtttatatactagtagcggccgctgcag

Prefix/Suffix
6xHis
Intimin’
SpyCatcherAmber
Double terminator

Part A. Testing expression levels of fusion proteins:

1. Intimin'-SpyTag
2. Intimin'-SpyCatcher

Experiments in brief:

1. Two cell lines expressing either fusion protein will be combined and will measure sedimentation levels or using a particle sizer
    a. Wild type + wild type - measure sedimentation
    b. Wild type + SpyTag/Catcher - measure sedimentation (after DNAcome)
2. Visualize protein localization using GFP tagged SpyTag or SpyCatcher added externally onto cells expressing surface binding partner (cell-protein interaction)
3. Visualize expression levels of each fusion protein using SDS PAGE gel
    a. Run wild-types through the SDS PAGE.
4. His tag purification of complex formed

Protein Expression protocol

Day n-1, Late afternoon
Set up starter culture:
10ml medium/antibiotic
1 colony from plate/glycerol stock
37 oC o/n

Day 1
Subculture o/n culture into fresh medium/antibiotic.
Volume: more than enough to split into later cultures.
Dilution: Add o/n culture to 1-2% final volume.
37 oC, 2h Measure OD600
Blank w clean medium.

Induce if mid-log phase. (Log phase OD600 = 0.4-0.8. Mid-log phase is around 0.6.) (If not yet log phase/mid-log phase, return culture to incubator, check again in 20-30mins.)

Split culture into -+ induction (or whatever conditions you're trying). (Uninduced control is pretty important.)
Add inducer to relevant concentration (eg. IPTG to 1 mM).
Incubate: 37 oC, 2-3 h or 30 oC, 3-6 h or 16-25 oC o/n

Harvest cells:
Take final OD.
Pellet 2.5 OD cells in cooled microfuge (usually around 1-2ml culture). (4min, 8,000 xg, 4oC)
- wash in 500 µl cold buffer*, re-pellet
- resuspend in lysis buffer*
*Buffers to use here will depend on application. You don't want the wash buffer to be a lysis buffer, so Tris-Cl of ~ pH 7.5 w NaCl between 50-150 mM is ideal.

Lysis buffer: can be like wash buffer if sonicating. Can also add reagents for lysis, eg. detergents like Tween or Triton. Most commonly I add lysozyme to 1mg/ml final concentration and incubate at for 30 oC, 20min.

50 mM Tris-HCl pH 7.5
100 mM NaCl
1 mM DTT (for intracellular proteins)
5% glycerol (possibly)

I keep the final buffer to 250 µl usually. This means you'll have 2.5OD cells/250 µl = 0.01 OD/µl, which is a good concentration, as 10 µl of this (0.1OD) is a decent # cells and a decent amount of proteins to load onto SDS-PAGE.

Lysis either:
(1) sonication: cup horn sonicator, settings 100% amplitude, 2min, 30s on/30s off.
(2) lysozyme (see above) - typically followed by sonication as well.
(3) can freeze pellet after washing (see above), and on thawing, either do sonication or lysozyme method.
Separation of fractions: spin in cooled microfuge: 30' at 16,000 xg, 4oC pellet will contain **insoluble proteins**, membrane fractions. **Soluble** material can be transferred to new eppie. pellet can be resuspended in 250 µl 1X LB.

Prep samples for gel: Soluble: 15 µl sample + 15 µl 2xLB [= 30 µl, of which to load 20 µl on gel] boil 95oC, 5min
Insoluble: boil whole 250 µl as above.

Day 2

Run SDS-PAGE gels to determine whether your protein was expressed.
Typically, MW ladder (NEB colour prestained/broad range, 5 µl), soluble fractions, insoluble fractions. Soluble: load 20 (of 1:1 sample:2xLB), Insoluble: load 5 µl (of sample in 1xLB).
*Insoluble fractions run better if they've been passed through a syringe to break up DNA.

Typically: 80-100 V until samples get through stacking gel, then 120V+. This takes ~2h total. (Technically possible to run it much faster, resolution is very poor though.)
Stain: instant blue, 30min RT on rocker. Destain in dH2O, 30min or o/n.
Capture on Licor

Part B. Testing covalent interaction between fusion proteins in vitro

SDS-PAGE to detect amide bond formation between SpyTag-fusion protein and SpyCatcher

Proteins mixed at 10uM in PBS pH7.4 at 24*C for 3hr (quantifications performed in triplicate)
Stop reaction by heating samples in SDS loading buffer on a Bio-Rad C1000 thermal cycler at 95 °C for 7 min.
SDS-PAGE on 14% polyacrylamide gels, at 200 V for approximately 1 h.
Gels stained with Instant Blue Coomassie stain (Triple Red Ltd.) and band intensities were quantified using a Gel Doc XR imager and Image Lab 3.0 software (Bio-Rad).

Reactions for pH-dependence: Mixing 10 μM of each protein in 40 mM Na2HPO4 with 20 mM citric acid pH 7.0 (phosphate-citrate) for the indicated time at 25 °C, or at the indicated temperature and pH. (PBS alone would not enable proper buffering over the pH range explored.)
For determining temperature dependence: all reactions were incubated in a Bio-Rad C1000 thermal cycler at 4, 25, and 37 °C with a heated lid to prevent evaporation.
To calculate the rate constant, SpyTag-MBP and SpyCatcher at 10 μM were mixed in triplicate in phosphate-citrate and incubated at 25 °C for 1, 3, or 5 min, in the linear part of the reaction. Samples were then heated to 95 °C for 7 min in SDS loading buffer and analyzed on 14% SDS-PAGE with Coomassie staining. Unreacted SpyCatcher concentration was quantified from band intensity as above. 1⁄[unreacted Spy Catcher] was plotted against time and a straight line, whose gradient corresponds to the second order rate constant, was fitted using the "LINEST" linear least squares curve-fitting routine in Excel. The units were converted from μM−1 min−1 to M−1 s−1.

→ could include GFP to see in vivo localization of fusion proteins

Part C. Testing covalent interaction between fusion proteins

- Combine cultures, one expressing SpyTag fusion protein (that had the highest expression levels) with another culture expressing the SpyCatcher fusion protein (which had the highest expression levels)
- Repeat protein expression protocol
- Isolate protein extracts using His column
  → ensure His tag is on SpyCatcher

Cell aggregation:
http://www.pnas.org/content/100/23/13259.full

Microscopy and Data Acquisition. To record the motion of individual cells in the clusters, the slides were left undisturbed for ≈20 min on the microscope stage, which was found to be the optimal time required to form tight clusters that did not drift. Images were acquired within the window of 20–40 min because after 40 min many cells lost motility and the clusters had a tendency to disperse. This result is probably caused by exhaustion of oxygen by bacterial respiration. Cells grown in LB medium typically loose motility under a coverslip after ≈20 min. The cluster size in LB medium is comparable to that observed in minimal media (M9 glycerol). Both dark field and fluorescence microscopy were used to characterize the macroscopic cluster properties and the motility of single cells. Images were acquired at 15 frames/s by using a Nikon E800 microscope equipped with a cooled CCD camera (CoolSNAP$_{HQ}$, Roper Scientific, Duluth, GA).
Data Analysis. Cells were tracked by using Metamorph (Universal Imaging Systems), which yielded the coordinates of each fluorescent cell for every frame of the video. Cells that left the field of view were not tracked. These data were then analyzed by using a tumble detection algorithm similar to Alon *et al.* (19) implemented in MATLAB (Mathworks, Natick, MA). Cells that swum at <75% of the mean speed were discarded from the data set. Success of the program was verified by visual examination of recordings and was found to be 90% accurate. The errors were largely due to cells that did not slow down while changing direction. It is possible that these may not in fact be tumble events but instead collisions with other cells or with the slide or coverslip. The center of a cluster was determined by averaging dark field images acquired over 6.5 s. In total, from the trajectories of 32 individual cells expressing GFP found in 28 clusters, 97 tumble events were registered. To determine the cell density profile, 19 clusters consisting only of cells expressing GFP were analyzed. The mean swimming speed of cells was determined to be 27 μm/s, which is the projected 2D speed. The actual speed will be a slightly greater because the system is only quasi-2D; i.e., the height of the system was ≈40 μm, which is approximately one-fifth of the typical cluster diameter.

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0107445
Fluorescence microscopy
For the selection lines derived from *E. coli* MGAY, when the cells reached an absorbance 0.2 (600 nm), they were placed on a slide that was layered with a 1.5% agarose pad and covered with a cover slip to immobilize the cells. Observations were made using differential interference contrast (DIC) and fluorescence imaging on Zeiss Axioimager M-1 upright microscope (100X/1.40 Oil DIC M27) with a color digital camera controlled by Axio software 4.8. Images both DIC/fluorescence were taken using exposure time of 30 millisecond illumination. The resulting images were processed and analyzed using Image J 1.44c software [25]. The fluorescence image alone was used for quantification of fluorescence intensity reflecting aggregate size whereas composite image formed by merging DIC and fluorescence was used to determine the location of protein aggregates in a cell.

Part D: Light induction

Introduce construct of best fusion protein partners in plasmid downstream of the light induction control promoter

1. Seed cultures from freshly transformed plates and grow overnight in 5 ml of LB medium supplemented with appropriate antibiotics at 37°C with shaking (225 rpm). Where specified, *N*-(3-oxo-hexanoyl)-l-HSL was added to the cultures at the indicated concentrations.
2. Overnight cultures were diluted into 5 ml of fresh pre-warmed M9 medium [(12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl), 1 M $MgSO_4$, 1 M $CaCl_2$, 0.2% (w/v) casamino acids and 20 mM glucose as a sole carbon source. As appropriate, antibiotics] for 2–3 h exponential outgrowth at 37°C with shaking (225 rpm).

3. Cultures aliquoted (0.5 ml) into 0.5 ml of pre-warmed M9 medium in triplicates of total 1 ml volume in a flat-bottom 12-well microplate (Nunc™). The microplate was incubated at 37°C with shaking (120 rpm) in a mini shaker incubator with illumination or kept in dark (wrapped in black cloth covering all edges) between each cycle of measurements.
4. Cells are illuminated using a custom built 3 × 4 LED blue light panel (465 nm) with adjustable ON/OFF pulsing and intensity for individual wells.

IF WE SOMEHOW HAVE GFP expression as well, we could observe directly junction between cells that have adhered, fluoresce

Time series OD (600 nm) and fluorescence (GFP: excitation 485 nm, emission 528 nm and RFP: excitation 540 nm, emission 600 nm) were read using Synergy™ HTX Multi-Mode Microplate Reader (BioTek). In all experiments, auto-fluorescence was measured using a negative control strain lacking the fluorescence reporter. The fluorescence/OD_{600} (Fluo/OD_{600}) at a specific time for a sample culture was determined after subtracting from each of the technical triplicate readings of the negative control cultures (fluorescence free) at the same time. The fluorescence synthesis rate (Fluo.$OD_{600}{}^{-1}$/min) of any sample at time t, was calculated by taking the difference of Fluo/OD_{600} values from two time points and dividing the result by the time interval δt. Normalization of Fluo/OD_{600} values was calculated into a new arbitrary range (min = 0) to (max = 1) with the observed original dataset min and max values.

Otherwise we would just perform protein expression assays post light induction vs no light induction and His tag column

24/07/17
Experiment:
Culture Preparation

Introduction:
Prepare the overnight culture for experiments on bacterial viabilities in lubricants and M9 media. The experiments are based on the irrE transformed and non-transformed cells. Another test is also done as reference on the original strain from which the plasmid was obtained.

Materials:
ON Culture 1 = NEB 10-ß irrE transformed cells (Positive) + LB + 20 µl Ampicillin
ON Culture 2 = NEB 10-ß irrE non-transformed cells (Negative, wild type) + LB
ON Culture 3 = irrE Stellar cells (plasmid original transformed strain) + LB +20 µl Ampicillin

20 ml each for 3 cultures.

Method:
Normal inoculation method.
Keep everything sterilized and culture is supposed to be autoclaved.
Take optical densities tomorrow.

Appendix:


Cell adhesion control experiments

Experiments in brief:

1. Two cell lines expressing either fusion protein will be combined and will measure sedimentation levels or using a particle sizer
    a. Wild type + wild type - measure sedimentation
    b. Wild type + SpyTag/Catcher - measure sedimentation (after cells come)
2. Visualize expression levels of each fusion protein using SDS PAGE gel
    a. Run wild-types through the SDS PAGE
3. Visualize protein localization using GFP tagged SpyTag or SpyCatcher added externally onto cells expressing surface binding partner (cell-protein interaction).
4. His tag purification of complex formed

31/07/17
Experiment:
Cell Growth against oxidative stress, high salinity environment and lack of nutrients.

Introduction:
Test the growth conditions of IrrE plasmid harboring cells and negative control in media with various stress. The oxidative stress is brought by hydrogen peroxide. The high salinity environment is created by high molarity NaCl solution. The M9 media is a good environment to test the cell growth in lack of nutrients.

The experiment can be referred to previous paper. Certain modification on the concentration of H_2O_2 and NaCl so that more data can be collected and be characterized. Both positive and negative control should have plasmid but positive has IrrE plasmid and negative has plain plasmid.

Materials:
Plate reader
ON culture of +/-
96 x well plates
Ampicillin (Depends on the vector)
LB broth

Methods: (+/-=control groups, 3= triplicates as biological repeats)
Take 1 ml of ON culture, measure OD_{600}

- **H_2O_2 + M9/LB**
    1. Pellet 1 ml cell culture and re-suspend with 1 ml LB with 15 µl of H_2O_2 for 10 mins
    2. Pellet and re-suspend with LB/M9
    3. Measure the optical density and calculate equivalent volume of OD_{600}=0.1
    4. Inoculate to 12 tubes (3+&3- in LB)/ (3+&3- in M9)

- **NaCl + M9/LB**
    1. Measure the optical density and calculate equivalent volume of OD_{600}=0.1
    2. Add NaCl to LB/M9 culture to 1 M.
    3. Inoculate to 12 tubes (3+&3- in LB)/ (3+&3- in M9)

- **M9/LB Media**
    1. Measure the optical density and calculate equivalent volume of OD_{600}=0.1
    2. Inoculate to 12 tubes (3+&3- in LB)/ (3+&3- in M9)

The results should provide sets of comparing data.

1. Growth in 3 stresses conditions. (Stress only variable and has the negative control all)
2. Cell growth in Pure M9 and LB as reference. (Both Negative and Positive)

Appendix:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2635966/

For M9 + Salt.

+

M9	M9 + H_3O_2	M9 + NaCl
① M9		
② M9	M9 H_3O_2	M9 + NaCl
③ M9	M9 + H_3O_2	M9 + NaCl

LB

① LB	LB H_3O_2	LB NaCl
② LB	LB H_3O_2	LB NaCl
③ LB	LB H_3O_2	LB NaCl

−

M9	M9 + H_3O_2	M9 NaCl
① M9		
② M9	M9 H_3O_2	M9 NaCl
③ M9	M9 H_3O_2	M9 NaCl

LB

① LB	LB H_3O_2	LB NaCl
② LB	LB H_3O_2	LB NaCl
③ LB	LB H_3O_2	LB NaCl

20 mL LB × 18

01/08/17
Experiment:
IrrE Positive and Negative Hydrogen Peroxide Resistance Preliminary Screening

Introduction:
To pre-test the viability of IrrE harbouring strains and negative control strain in the presence of 1.5% and 1.0% of H_2O_2. This provides the information for the culturing experiments in next session.

Materials:
LB
H_2O_2
Agar plates and agar plates with 100 µg/ml Ampicillin
Spreaders

Methods:

1. Take the ON culture of both strains

Centrifuge 3 tubes of 1 ml of each, labelled as LB, LB+1.0% H_2O_2
, LB+1.5% H_2O_2. Do the same labelling on negative ones.

2. Re-suspend 2 tubes labelled as LB with fresh LB

Re-suspend 2 tubes labelled as LB+1.0% H_2O_2 with 990 µl of LB and 10 µl of H_2O_2

3. Re-suspend 2 tubes labelled as LB with fresh LB

Re-suspend 2 tubes labelled as LB+1.0% H_2O_2 with 985 µl of LB and 15 µl of H_2O_2

4. Incubate all tubes in 37˚C water bath for 10 mins
5. Take 4 tubes with H_2O_2 and centrifuge. Then re-suspend with fresh LB

4 Plates are used, labelled as: (with quarters) Q = Quarter
+ Group Amp 1.0%: 1stQ = LB 2ndQ= LB+1.0% H_2O_2 3rdQ=1/10 LB+1.0% H_2O_2 4thQ = N/A
+ Group Amp 1.5%: 1stQ = LB 2ndQ= LB+1.5% H_2O_2 3rdQ= 1/10 LB+1.5% H_2O_2 4thQ = N/A
- Group Agar 1.0%: 1stQ = LB 2ndQ= LB+1.0% H_2O_2 3rdQ= 1/10 LB+1.0% H_2O_2 4thQ = N/A
- Group Agar 1.5%: 1stQ = LB 2ndQ= LB+1.5% H_2O_2 3rdQ= 1/10 LB+1.5% H_2O_2 4thQ = N/A

Plate as labelled accordingly.
Incubate the plates ON to see the colonies.

25/07/17
Experiment:
Cell Growth in M9 media and Lubricant

Introduction:
Monitor the growth of irrE cells in different media by measuring the optical densities. The cells are cultures for 8hrs. Measurements are taken every 20 mins when the cells duplicate.

Materials:
ON culture 1 2 3
12 Shaking flasks
Pipette gun
Lubricant
M9 solution
LB media

Method:
Take optical densities of each ON culture and dilute them into same cell density.

Prepare 50ml of culture 1/2/3/4
1= M9 solution
2= LB media
3= Lubricant + LB media (Lubricant concentration 40%v/v)
4= Lubricant + M9 solution (Lubricant concentration 40%v/v)

Label 12 flasks as below
- (+) Culture 1/2/3/4
- (-) Culture 1/2/3/4
- (s+) Culture 1/2/3/4 12 flasks in total.

Inoculate accordingly into each flask.
The number of cells inoculated must be same for three cultures.
The method is described below:
The Equation was $C_1V_1=C_2V_2$, the concentration (C) and volume (V) before and after dilution.
OD_{600} of ON culture 1 2 3 (1/8 dilution)
C_1=0.154*8=1.232 C_2V_2=0.5
C_2=0.160*8=1.280 C_2V_2=0.5
C_3=0.214*8=1.712 C_2V_2=0.5
After inoculating
Take optical density of 12 cultures every 20 mins for 8 hours.

Appendix:
Data was saved on the Excel in the same folder.


New Brunswick Scientific

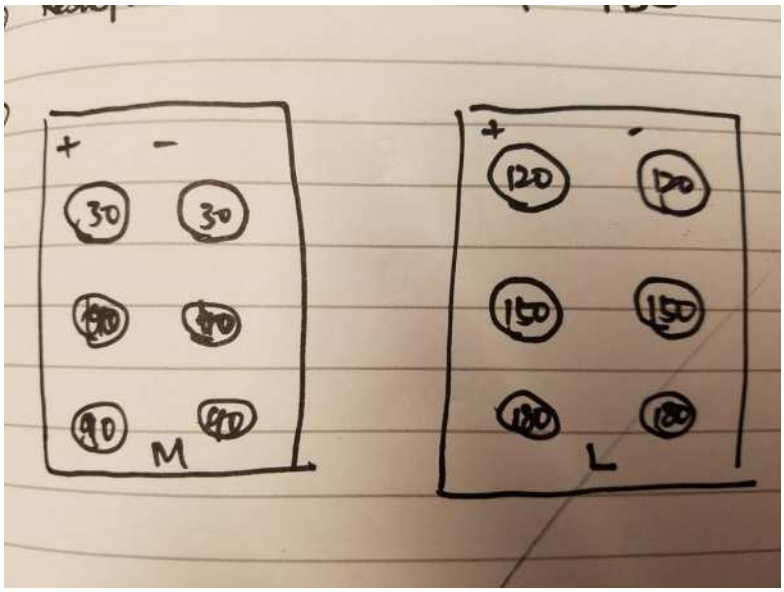
12/07/17
Experiment:
irrE Exposure Experiments III

Introduction:
Test the viability of cells transformed with irrE plasmids (+) as positive group with the comparison to non-transformed ones (-). The viabilities of two groups are compared against period of exposure. The overnight culture was prepared in advance. Additionally, the plated is covered with aluminum to prevent *E.coli* photo-reactivation and another reference group is un-covered.

Materials:
Sterile 6-well plates
ON culture of both Positive and negative groups.
Autoclaved Spreaders
LB Plates
LB Plates with Ampicillin
Sterile PBS solution

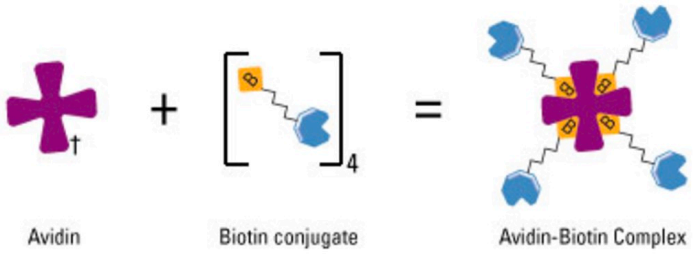
Methods:
Sterilize everything used involving cells.
Centrifuge 2 ml of ON culture of each and re-suspend with PBS.
Take out 5 µl of both resuspension cultures.
Pipette cell culture onto the according wells. (as shown in the appendix)
Re-suspend with 1995 µl of PBS.
Expose the plates in the UV cross-linker for the period required.
Both culture re-suspensions are exposed for 30/60/90/120/150/180 mins and get covered with aluminum foil.
Prepare one plate for UV exposed but not uncovered as reference. The Exposure time is 90 mins.
Take 20 µl of the culture after exposure and plate it onto plates with according antibiotics.
Repeat the steps when exposure period increases.

Appendix:

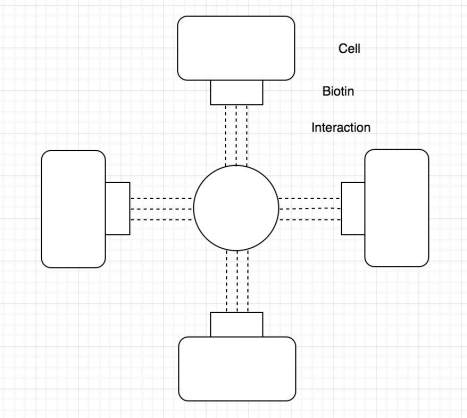

+
-
30
30
90
90
M
+
-
120
120
150
150
180
180
L

20/09/17
Experiment: Cell Adhesion by Biotin-Avidin Conjugation

Experiment Design:
Avidin has a high affinity to biotin, one Avidin molecule can bind up to 4 biotin molecules, which are normally conjugated to an enzyme, antibody or target protein to form an avidin-biotin complex.



By this design, we can apply similar idea to *E.coli* cells. Once cells are biotinylated and biotin molecules are attached to the surface of the cells, the cell-biotin conjugate will be able to bind to avidin once avidin is added. Cell adhesion is achieved.
For tetrameric avidin, the theoretical maximum is 4 moles biotin:1 mole Avidin [1]
There is no way to see how many cells have been biotinylated. Two experiments of different Avidin Biotin-cell ratio are used in the experiment with a control.
Measurements to be discussed.



Materials:
Sulfosuccinimidobiotin, NHS-Biotin Solution, Dimethylformamide(DMF), Ice-cold PBS solution (pH 8.0), *E.coli* pUC 19 Overnight culture , 25˚C Waterbath, Glycine, Avidin and Measurement instruments.

Lab-based Methods:

A. Cell Biotinylation [2]
    1. Wash cells three times with ice-cold PBS (pH 8.0) to remove amine-containing culture media and proteins from the cells. (6000rpm 5 mins each time)
    2. Suspend cells at concentration of OD_{600}= 0.032 in PBS.
    3. 20mM NHS-Biotin solution (4-5mg to 0.5ml DMF).
    4. Add 100µl solution to 1ml of cell suspension and incubate for 30 mins at room temperature.

5. Wash cells with PBS+100mM Glycine to remove excess biotin

B. Biotin-Avidin System

Avidin is very soluble both in water and salt solutions so avidin is going to be dissolved in PBS solution to required molarity.

1. Control: Cells are re-suspended in 100µl of 20mM NHS-Biotin solution and mixed with PBS as control.
2. 1:1: Cells are re-suspended in 100µl of 20mM NHS-Biotin solution and mixed with 100µl 20mM Avidin solution.
3. 1:4: Cells are re-suspended in 100µl of 20mM NHS-Biotin solution and mixed with 25µl 20mM Avidin solution.

Following procedures depend on how measurements are made.

C. Part 3-Cell adhesion measurement

To be discussed

[1] https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/a9275pis.pdf

[2]https://tools.thermofisher.com/content/sfs/manuals/MAN0011206_EZ_NHS_Biotin_Reag_UG.pdf

22/09/17
Bacterial Cell Aggregation by Biotin-Avidin adhesion

Materials:
Biotin: 50 mg ✔
Avidin: 2mg✔
Glycine: >2 g✔
BL21(DE3) pET14b-GFP (Amp): 50mL✔
BL21(DE3) pL4SS-mCherry (Cam): 50mL✔
Autoclaved LB Media: 500 mL✔
Autoclaved Baffled Shaking Flasks with cotton: 2
Fume Hood
Procedures:

A. Solution Preparation (On Wednesday)
    1. 30 mL of 100mM Glycine: 225 mg of glycine in 30mL PBS (Filter glycine solution before use)
    2. Add 2 mg Avidin to 1 mL PBS (0.0312mM Avidin)(3mg left)
    3. Add 8.868 mg Biotin to 1ml DMF (10mM Biotin)
B. Cell Preparation (Ready until Wednesday)
    1. Inoculate both strains onto according plates and incubate at 37˚C overnight✔
    2. Take out 2 plates and put them into 4˚C freezer (Friday) ✔
    3. Pick in total of 5-10 colonies (on one inoculation loop) and inoculate into 10ml LB. ✔
    4. Incubate overnight at 37˚C at 160rpm✔
    5. Prepare 50mL LB with according antibiotics in 250mL baffled shaking flasks (Tuesday)
    6. Inoculate 100µL of overnight culture(Tuesday)
    7. Induce with IPTG when OD_{600} =1 (Tuesday)
    8. Put cultures at room temperature overnight shaking at 160 rpm.
    9. Proceed same procedure with the other strain. Overnight culture OD_{600} >5.0
C. Biotinylation
    1. Wash 20mL of cells three times with ice-cold PBS (pH 8.0) at 5000rpm 5 mins each time
    2. Measure OD600 and adjust to 1
    3. Add 100µl of 10mM NHS-Biotin to 900µL of cells and flick the tube 5-6 times to mix (Repeat for all samples except for the un-biotinylated cells)
    4. Incubate cells in room temperature for at least 1 hour
    5. Wash cells in 1mL Glycine solution to remove excess biotin
    6. Re-suspend cells to OD_{600}=1.0 with 1 mL PBS
D. Adhesion
    For filming aggregation, start with OD_{600}=1.0
    For microscopy and sedimentation with plating, start with OD_{600}=1.0
    Experiment groups: (Repeat same procedure with the other strain)
    Do the experiment on the most fluorescent cells.

Groups	Cells	Biotin	Avidin	DMF	PBS	
Control 1	1mL	-	100µl 0.0312mM	100µl	-	
Control 3	1ml	-	-	100µl	100µl	
Control 2	1mL	100µl 1.0 mM	-	-	100µL	
4:1	1mL	100µl 0.125 mM	100µl 0.0312mM	-	-	
X:1(Excess Biotin)	1mL	100µl 1.0 mM	100µl 0.0312mM	-	-	Excess Biotin
		Control 1	Un-Biotinylated			
		Control 2	Un-Avidinated			
		Control 3	Unlabelled and un-avidinated			

E. Incubation
    1. Incubate all vials of cells at 25˚C water bath for 1 hour.
    2. After incubation, aggregation should have started and measurements should be taken at first time point when they are taken out of the water bath.
    3. Put vials at benchtop for 5 hours. Measurement intervals stated below.
F. Measurements
    1. Film sedimentation
       Use normal phone to focus at the tube, film for 5 hours. (or less)
    2. Natural Sedimentation, plate and count colonies
        1. Let all groups to be settled down. Mark a point for consecutive measurements.
        2. Take out 10 µL of supernatant of the culture FROM SAME POINT. Time interval: 30 mins, 1hour, 2hours, 3hours, 5hours.
        3. Re-suspend 10 µL in 90 µL of LB (x10 dilution)
        4. Plate onto according antibiotics containing plates
        5. Incubate at 37˚C overnight
    3. Cytometric Microscopy
        1. Take 10µL of the initial culture
        2. Observe under wide-field microscope

26/09/17
Bacterial Cell Aggregation by Biotin-Avidin adhesion

Materials:
Nhs Biotin: 50 mg □
Avidin: 2mg□
Glycine: >2 g□
BL21(DE3) pET14b-GFP (Amp): 50mL□
BL21(DE3) pL4SS-mCherry (Cam): 50mL□
Autoclaved LB Media: 500 mL□
Autoclaved Baffled Shaking Flasks with cotton: 2
Fume Hood
Procedures:

1. **Preparation**
    1. Prepare 50 mL of 100mM Glycine: 375 mg of glycine in 50mL PBS (Filter glycine solution before use)
    2. Add 2 mg Avidin to 1 mL PBS (0.0312mM Avidin)(3mg left)
    3. Add 8.868 mg Biotin to 1ml DMF (10mM Biotin) **– dilute to 1mM?**
    4. Prepare 2x 250mL baffled conical flasks, each containing 50m LB with appropriate antibiotics
    5. Prepare 2x 50mL LB with appropriate antibiotics in 50mL Falcon tubes
    6. Prepare 500mL PBS. Filter sterilize and store in 4˚C fridge.
    7. Prepare an appropriate tube rack and lighting setup for 5 hour video capture of cell aggregation in tubes.

2. **Cell Preparation**
    1. Streak both strains onto plates with appropriate antibiotic and place in static incubate at 37˚C overnight□
    2. Move plates to 4˚C freezer for overnight storage. Seal with nesco film. □
    3. Pick in total of 10 colonies (on one inoculation loop) into 10ml LB with appropriate antibiotic. □
    4. Incubate overnight at 37˚C at 160rpm□
    5. 9am Use 100µL of each culture to inoculate 50m LB with with appropriate antibiotics in 250mL flasks.
    6. Incubate at 37˚C at 160rpm. Monitor OD hourly and add X volume of Y conc. IPTG when OD_{600} =1 then leave incubation to continue overinight.

3. **Biotinylation (for both strains)**
    1. Move 20mL of culture from conical flask to 50mL Falcon tube.
    2. Cf at 5000rpm for 5 mins
    3. Discard supernatant and resuspend pellet in 20mL ice-cold PBS (pH 8.0)
    4. Repeat above 3 steps three times.
    5. Measure OD600 of final PBS / cell solution.
    6. Dilute an aliquot of the PBS / cell solution of the necessary volume to result in an 8mL PBS / cell solution of OD600 = 1.

Biotinylation continued.

7. Transer 900µL of the OD600 = 1 PBS / cell solution to each of eight 1.5mL Eppendorf tubes, labelled A-H.
8. Add the solutions indicated in Table 1 'Step 1 Biotinylation' to each of the tubes, labelled A-H.
9. Incubate all tubes for 1 hour at Room Temp.
10. Cf tubes at XXX RPM. Discard supernatant.
11. Resuspend pellet in 1mL Glycine.
12. Cf tubes at XXX RPM. Discard glycine supernatant.
13. Resuspend pellet in 900µL PBS.

4. Avidin incuabation

14. Add the solutions indicated in Table 2 'Step 2 Avidin incuabation' to each of the tubes. **Take a note of the time.** Gently swirl tubes 3 times.
15. Proceed immediately to aggregation measurement.

5. Aggregation measurements

1. Transfer 500 µL of cell solution gently into a second, labeled tube for video recording.
2. Place these new tubes in an appropriate tube rack and lighting setup for 5 hour video capture of cell aggregation.
3. Place 40x 1.5mL Eppendorf tubes in a rack. Add 90 µL PBS to each tube.
4. Each hour for 5 hours, remove 10µL FROM THE UPPER SURFACE of the culture and add to 90 µL PBS in a separate tube. Transfer what is now a 100µL to a LABELLED LB agar plate, containing appropriate antibiotic, and spread. Place plate in static incubator overnight. Log approx. colony counts the next day.
5. Use the remaining 500 µL of cell solution from step 1. above for microscopy studies with Steffi Frank.

Table 1

	Step 1 Biotinylation					Step 2 Avidin Incubation	
Tube Label	Vol. OD = 1 PBS cell solution	1000µM nhs-Biotin	125µM nhs-Biotin	PBS	DMF	32µM Avidin	PBS
A	900µL				100µl	100µl	
B	900µL				100µl		100µl
C	900µL			100µL		100µl	
D	900µL			100µL			100µl
E	900µL		100µL			100µl	
F	900µL		100µL				100µl
G	900µL	100µL				100µl	
H	900µL	100µL					100µl

3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Avidin from egg white
BioUltra

Catalog Number **A9275**
Storage Temperature 2–8 °C

CAS RN 1405-69-2

Product Description

In the late 19th century and the 1910's, several reports indicated that feeding large quantities of dried egg white to animals produced a nutritional deficiency.[1,2] Administration of vitamin H, also known as biotin, remedied this deficiency. Eventually, it emerged that this deficiency resulted from the binding of biotin to a protein in egg white.[3,4] This protein was called "avidin", after its "avidity" for biotin.

Avidin is a tetrameric glycoprotein with an approximate molecular mass of 66–67 kDa.[5] It is composed of four subunits with each subunit containing 128 identical amino acid residues and a variable carbohydrate moiety.[5,6] The subunits may vary slightly in molecular mass due to the carbohydrate composition. Only Asp^{17} is glycosylated.[5,7] The carbohydrate moiety can have at least three different carbohydrate structural types.[8] Avidin can be dissociated into subunits under strongly denaturing conditions. Each subunit is separately capable of binding biotin with a dissociation constant (K_d) = 10^{-7} M.[6,9]

The avidin-biotin association constant (K_a = 10^{15} M^{-1}) is one of the strongest affinities known. The complex is stable to 100 °C, and significantly stable to detergents and denaturants.[10] Avidin is stable to ~85 °C without biotin. Biophysical studies of the avidin-biotin complex have implicated particular tryptophan and lysine residues in the biotin-binding site of avidin.[11,12] The crystal structure of avidin has been published.[13] The crystal structure of a deglycosylated form of avidin complexed with biotin has been reported.[14]

Because of the stability of both avidin and biotin, each of these molecules has been used as "labels" for antibodies, fluorescent dyes, proteins, and other molecules of interest to biochemists. Avidin and biotin have each been incorporated into immobilized matrices. (The only way that monomeric avidin can exist is through its attachment to an agarose support.[9])

This product (A9275) is purified using affinity chromatography. It is dialyzed extensively against deionized water before being lyophilized. The basic procedures are modified from literature preparations.[13,15,16] This product is sold by protein content (determined by $E^{1\%}_{280}$).

Unit Definition: One unit will bind 1.0 μg of d-biotin.

Unit activity: ≥10 units per mg protein

Isoelectric point (pI):[8,9] 10

K_d for the avidin-biotin complex:[6] 10^{-15} M (neutral pH)

Binding capacity: For tetrameric avidin, the theoretical maximum is 4 moles biotin:1 mole avidin[6,13] or ~15 μg biotin/mg protein.[14]

Reported fluorescent wavelength:[9]
338 nm (avidin)
328 nm (avidin-biotin complex)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Avidin is very soluble both in water, up to 20 mg/mL,[8] and in salt solutions. Avidin solutions are stable over a wide range of pH and temperatures, particularly when combined with biotin.[6,17] Avidin can be crystallized from ammonium sulfate at >2.5 M at pH 5.[13] Since one tryptophan residue per subunit is involved in the binding site, avidin can be inactivated by oxidizing agents such as ozone, peroxide, or strong light.[11] Solutions should be stored at –20 °C.

Storage/Stability

The avidin-biotin complex is even more heat stable than avidin alone:

- It is only 10% dissociated after 15 minutes at 100 °C.[6] It is not completely dissociated after 60 minutes at 100 °C.
- The complex can be quantitatively dissociated only under autoclaving conditions, e.g. 120 °C, 15 minutes.[15]

When avidin was reduced in the presence of 9 M urea, its biotin-binding activity was unchanged. The protein was denatured and lost biotin-binding activity as the pH was gradually lowered to pH 1. However, when the pH was raised to pH 3, avidin regained native configuration and binding activity. The complex is also extremely stable at high pH, being only 20% ionized even at pH 13.[17]

References

1. Bateman, W.G., *J. Biol. Chem.*, **26(1)**, 263-291 (1916).
2. György, P., *J. Biol. Chem.*, **131(2)**, 733-744 (1939).
3. du Vigneaud, V., *et al.*, *J. Biol. Chem.*, **140(2)**, 643-651 (1941).
4. György, P., *Science*, **93(2420)**, 477-478 (1941).
5. DeLange, R.J., and Huang, T.-S., *J. Biol. Chem.*, **246(3)**, 698-709 (1971).
6. Korpela, J., *Med. Biol.*, **62(1)**, 5-26 (1984).
7. Woolley, D.W., and Longsworth, L.G., *J. Biol. Chem.*, **142(1)**, 285-290 (1942).
8. Bruch, R.C. and White, H.B., III, *Biochemistry*, **21(21)**, 5334-5341 (1982).
9. Green, N.M., *Adv. Protein Chem.*, **29**, 85-133 (1975).
10. Ross, S.E., *et al.*, *BioTechniques*, **4**, 350-354 (1986).
11. Green, N.M., *Biochem. J.*, **89(3)**, 599-609 (1963).
12. Gitlin, G., *et al.*, *Biochem. J.*, **242**, 923-926 (1987).
13. Green, N.M., and Toms, E.J., *Biochem. J.*, **118(1)**, 67-70 (1970).
14. Livnah, O., *et al.*, *Proc. Nat. Acad. Sci. USA*, **90(11)**, 5076-5080 (1993).
15. Green, N.M., *Methods Enzymol.*, **XVIII (Part A)**, 414-417 (1970).
16. Melamed, M.D. and Green, N.M., *Biochem. J.*, **89(3)**, 591-599 (1963).
17. Green, N.M., *Biochem. J.*, **89(3)**, 609-620 (1963).

CKV,GCY,MAM 03/17-1

INSTRUCTIONS

EZ-Link® NHS-Biotin Reagents

20217 21336 21343

0237.4

Number **Description**

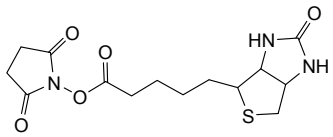
20217 **EZ-Link® NHS-Biotin,** 100 mg, *N*-hydroxysuccinimidobiotin

Molecular Weight: 341.38

Spacer Arm Length: 13.5 Å

Net Mass Added: 226.08

Storage: Upon receipt store desiccated at room temperature.



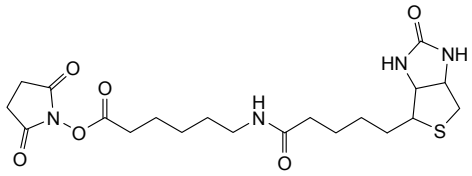
21336 **EZ-Link® NHS-LC-Biotin,** 50 mg, succinimidyl-6-(biotinamido)hexanoate

Molecular Weight: 454.54

Spacer Arm Length: 22.4 Å

Net Mass Added: 339.16

Storage: Upon receipt store desiccated at 4°C. Product is shipped at ambient temperature.



21343 **EZ-Link® NHS-LC-LC-Biotin,** 50 mg, succinimidyl-6-(biotinamido)-6-hexanamido hexanoate

Molecular Weight: 567.70

Spacer Arm Length: 30.5 Å

Net Mass Added: 452.24

Storage: Upon receipt store desiccated at 4°C. Product is shipped at ambient temperature.

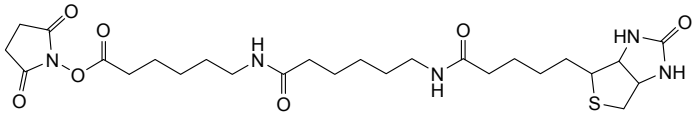


Table of Contents

Introduction

EZ-Link® NHS-Biotin Reagents enable simple and efficient biotin labeling of antibodies, proteins and any other primary amine-containing macromolecules in solution. Differing only in spacer arm lengths, the three reagents offer researchers the possibility of optimizing labeling and detection experiments where steric hindrance of biotin binding is an important factor.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermo.com/pierce

Biotin is a small naturally occurring vitamin that binds with high affinity to avidin and streptavidin proteins. Because it is so small (244 Da), biotin can be conjugated to many proteins without altering their biological activities. Labeled proteins may be purified from unlabeled proteins using immobilized streptavidin and avidin affinity gels (see Related Thermo Scientific Products), and they may be detected easily in ELISA, dot blot or Western blot applications using streptavidin or avidin-conjugated probes.

N-Hydroxysuccinimide (NHS) esters of biotin are the most popular type of biotinylation reagent. NHS-activated biotins react efficiently with primary amino groups (-NH_2) in pH 7-9 buffers to form stable amide bonds. Proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide that are available as targets for labeling with NHS-activated biotin reagents. Several different NHS esters of biotin are available, with varying properties and spacer arm lengths. The three EZ-Link® NHS-Biotin Reagents are not directly water soluble and must be dissolved in organic solvents such as DMSO or DMF before addition to aqueous solutions at the final concentration for the labeling reaction.

Biotinylation of intact cells has emerged as an important tool for studying the expression and regulation of receptors and transporters, differentiation of plasma membrane proteins from those localized to organelle membranes, and distribution of membrane proteins in polarized epithelial cells. Sulfo-NHS-Biotin reagents (see Related Thermo Scientific Products) do not readily permeate cell membranes and are commonly used for specifically labeling the cell surface. By contrast, NHS-Biotin reagents are membrane permeable and may be used to biotinylate proteins inside intact cells. Parallel experiments with NHS- and Sulfo-NHS-Biotin analogs may help to localize particular proteins of interest.

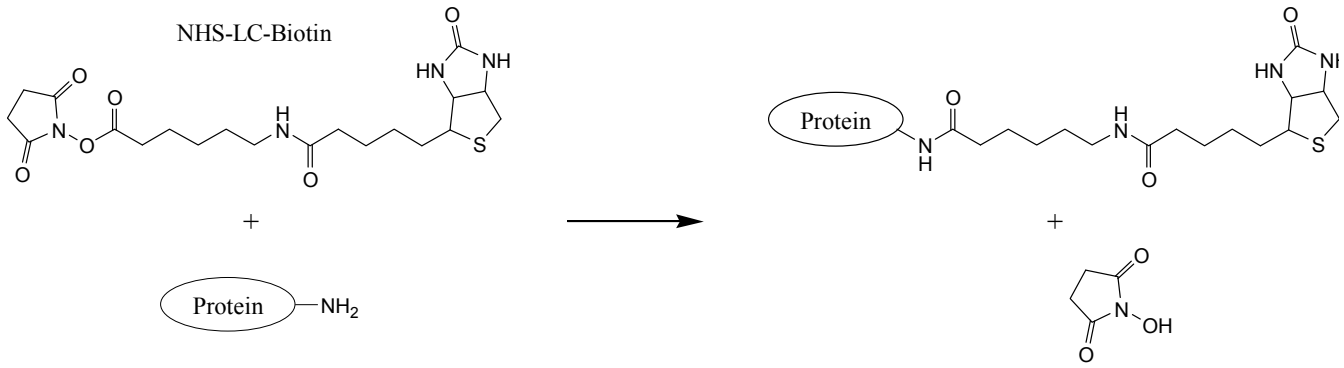


Figure 1. Reaction of NHS-LC-Biotin with primary amine. If drawn to scale, the oval representing the protein would be many times larger than the structures shown in this scheme and would likely contain several amino groups, each of which would be labeled in some proportion of the protein molecules in the reaction. Note that NHS is a leaving group (byproduct) in the reaction; this leaving group as well as any nonreacted biotin reagent is removed during the final desalting step in the procedure.

Important Product Information

- NHS-Biotin reagents are moisture-sensitive. If the vial of reagent has been stored cold, fully equilibrate vial to room temperature before opening to avoid moisture condensation inside the container.
- As directed in the procedure, dissolve the biotin reagent immediately before use. The NHS ester moiety readily hydrolyzes and becomes nonreactive; therefore, weigh and dissolve only a small amount of the reagent at a time, and do not prepare stock solutions for storage. Discard any unused reconstituted reagent.
- Avoid buffers containing primary amines (e.g., Tris or glycine) as these will compete with the reaction (see Figure 1). If necessary, dialyze or otherwise desalt to exchange the protein sample into an amine-free buffer such as phosphate buffered saline (PBS; see Related Thermo Scientific Products).

- When biotinylating proteins in solution, excess non-reacted biotin and reaction byproducts are easily removed by size exclusion using either desalting columns or dialysis (See Additional Information and Related Thermo Scientific Products). A 10 ml desalting column is best suited for processing biotinylation reactions involving 1-10 mg of protein in approximately 0.5-2 ml. For smaller amounts of protein and/or smaller reaction volumes, both the biotinylation reaction and subsequent buffer exchange may be performed in a single Slide-A-Lyzer® MINI Dialysis Unit. For larger reaction volumes than can be processed with a desalting column, either split the sample between two columns or use an appropriate Slide-A-Lyzer® Dialysis Cassette for buffer exchange steps.

Additional Materials Required

- Water-miscible organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF)
- Phosphate Buffered Saline (PBS) or other amine-free buffer having pH 7-8 for use as reaction buffer (see Important Product Information and Related Thermo Scientific Products)
- Desalting columns or dialysis units for buffer exchange (see Important Product Information and Related Thermo Scientific Products)

Procedure for Biotinylating Proteins

The following procedure ordinarily will yield incorporation of 3-5 biotins per molecule of protein. Antibodies, which are large proteins, often will label with ~8-12 biotin molecules per molecule of IgG, especially when greater molar excesses of biotin reagent are used (see Calculations). The molar ratio of biotin reagent to protein may be adjusted to obtain the level of incorporation desired.

A. Calculations

The amount of biotin reagent to use for each reaction depends on the amount of protein to be labeled and its concentration. By using the appropriate molar ratio of biotin to protein, the extent of labeling can be controlled. When labeling more dilute protein solutions, a greater molar fold excess of biotin is necessary to achieve the same results. Generally, for best results use ≥ 12-fold molar excess of biotin for a 10 mg/ml protein solution or ≥ 20-fold molar excess of biotin for a 2 mg/ml protein solution.

1. Calculate millimoles of biotin reagent to add to the reaction for a 20-fold molar excess:

$$\text{ml protein} \times \frac{\text{mg protein}}{\text{ml protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{\text{20 mmol Biotin}}{\text{mmol protein}} = \text{mmol Biotin}$$

   - 20 = Recommended molar fold excess of biotin for 2 mg/ml protein sample

2. Calculate microliters of 10 mM biotin reagent solution (prepared in Step B.3) to add to the reaction:

$$\text{mmol Biotin} \times \frac{\text{1,000,000 µl}}{\text{L}} \times \frac{\text{L}}{\text{10 mmol}} = \text{µl Biotin}$$

Example Calculation:

For 1 ml of a 2 mg/ml IgG (150,000 MW) solution, ~27 µl of 10 mM biotin reagent will be added.

$$\text{1ml IgG} \times \frac{\text{2 mg IgG}}{\text{1ml IgG}} \times \frac{\text{1mmol IgG}}{\text{150,000 mg IgG}} \times \frac{\text{20 mmol Biotin}}{\text{1mmol IgG}} = \text{0.000266 mmol Biotin}$$

$$\text{0.000266 mmol Biotin} \times \frac{\text{1,000,000 µl}}{\text{L}} \times \frac{\text{L}}{\text{10 mmol}} = \text{26.6 µl Biotin Reagent}$$

B. Biotin Labeling Reaction

1. If the biotin reagent has been stored cold, remove the vial from storage and fully equilibrate it to room temperature before opening in step 3.
2. Dissolve 1-10 mg protein in 0.5-2.0 ml PBS according to the calculation made in section A.

   Note: Protein that is already dissolved in amine-free buffer at pH 7.2-8.0 may be used without buffer exchange or dilution with PBS. Proteins in Tris or other amine-containing buffers must be exchanged into a suitable buffer.
3. Immediately before use, prepare a 10 mM solution of the biotin reagent in an organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF):
   - For NHS-Biotin (Product No. 20217), dissolve 2.0 mg reagent in 590 µl of solvent.
   - For NHS-LC-Biotin (Product No. 21336), dissolve 2.3 mg reagent in 500 µl of solvent.
   - For NHS-LC-LC-Biotin (Product No. 21343), dissolve 2.0 mg reagent in 350 µl of solvent.
4. Add the appropriate volume (see Calculations in section A) of 10 mM biotin reagent solution to the protein solution.
5. Incubate reaction on ice for two hours or at room temperature for 30 minutes.

   Note: Other than the possibility of ordinary protein degradation or microbial growth, there is no harm in reacting longer than the specified time.
6. Protein labeling is complete at this point, and although excess non-reacted and hydrolyzed biotin reagent remains in the solution, it is often possible to perform preliminary tests of the labeled protein by ELISA or Western blot. Once proper function and labeling of the protein has been confirmed, the labeled protein may be purified for optimal performance and stability using desalting or dialysis. If the level of biotin incorporation will be determined using the EZ™ Biotin Quantitation Kit (HABA assay; see Related Thermo Scientific Products), the protein first must be desalted or dialyzed to remove non-reacted biotin.

Procedure for Biotinylating Cells

Many variations of this procedure exist in the literature. Sulfo-NHS-Biotin reagents (see Related Thermo Scientific Products) do not readily permeate cell membranes and are commonly used for specifically labeling the cell surface. By contrast, NHS-Biotin reagents are membrane permeable and may be used to biotinylate proteins inside intact cells. Parallel experiments with NHS- and Sulfo-NHS-Biotin analogs may help to localize particular proteins of interest.

Labeling may be performed on cells in suspension or on adherent cells in culture plates. In the latter situation, diffusion of the NHS-Biotin reagent to all surfaces of the cells will be limited, and labeling will occur predominately on and through the exposed surface. Culture media must be washed from the cells, or amine-containing components will compete and quench the reaction to cell proteins. Using a more concentrated cell suspension is most effective since less biotin reagent will be required in the reaction. Generally, a final concentration of 2-5 mM NHS-Biotin reagent is effective. NHS-Biotin reactions occur more rapidly at higher pH; therefore, pH 8.0 is used in the following example so that labeling can be completed as quickly as possible.

1. Wash cells three times with ice-cold PBS (pH 8.0) to remove amine-containing culture media and proteins from the cells.
2. Suspend cells at a concentration of ~25 × 10^6 cells/ml in PBS (pH 8.0).
3. Prepare a 20 mM solution of NHS-Biotin reagent by dissolving 4-5 mg of reagent per 0.5 ml of water-miscible organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF).
4. Add 100 µl of NHS-Biotin reagent solution to each 1 ml of cell suspension (results in ~2 mM biotin reagent).
5. Incubate reaction mixture at room temperature for 30 minutes.

   Note: Longer reaction time may be necessary to ensure significant diffusion of NHS-Biotin reagent into the cells; otherwise, most labeling may occur at the cell surface.
6. Wash cells three times with PBS + 100 mM glycine to quench and remove excess biotin reagent and byproducts.
7. Lyse and/or analyze biotin-labeled cells as required for the research method.

Additional Information

Visit the web site for additional information related to this product, including the Tech Tip procedure titled "Perform labeling and other reactions in Slide-A-Lyzer® Dialysis Cassettes."

Related Thermo Scientific Products

21217	**EZ-Link® Sulfo-NHS-Biotin,** 50 mg, sulfosuccinimidobiotin
21335	**EZ-Link® Sulfo-NHS-LC-Biotin,** 100 mg, sulfosuccinimidyl-6-(biotin-amido)hexanoate
21338	**EZ-Link® Sulfo-NHS-LC-LC-Biotin,** 50 mg, sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate
28372	**BupH™ Phosphate Buffered Saline Packs,** 40 pack, each pack yields 500 ml of 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2 when reconstituted with 500 ml water.
69576	**Slide-A-Lyzer® MINI Dialysis Unit Kit,** for 10-100 µl sample volumes, 10 units plus float
66382, 66807	**Slide-A-Lyzer® Dialysis Cassette Kits,** for 0.5-3 ml and 3-12 ml sample volumes, respectively
89889	**Zeba™ Desalt Spin Columns,** 5 × 2 ml columns, for desalting 200-700 µl samples
89891	**Zeba™ Desalt Spin Columns,** 5 × 5 ml columns, for desalting 500-2,000 µl samples
28005	**EZ™ Biotin Quantitation Kit,** HABA assay kit to determine levels of biotin incorporation
21450, 21440	**EZ-Link® NHS-PEO$_4$-Solid Phase Biotinylation Kit**, one-step antibody labeling and purification
20347	**Immobilized Streptavidin Gel**, 2 ml
20228	**Immobilized Monomeric Avidin Kit**, bind and gently elute biotin-labeled molecules
21126	**Streptavidin, Horseradish Peroxidase Conjugated**, 1 mg

Product References

Bessho, Y., *et al.* (2002). A tRNA aminoacylation system for non-natural amino acids based on a programmable ribozyme. *Nat. Biotechnol.* **20:**723-8.

Bruneau, N., *et al.* (2003). Lectin-like Ox-LDL receptor is expressed in human INT-407 intestinal cells: involvement in the transcytosis of pancreatic bile salt-dependent lipase. *Mol. Biol. Cell* **14:**2861-75.

Fouassier, L., *et al.* (2000). Evidence for Ezrin-Radixin-Moesin-binding phosphoprotein 50 (EBP50) self-association through PDZ-PDZ interactions. *J. Biol. Chem.* **275(32):**25039-45.

Sélo, I., *et al.* (1996). Preferential labeling of α-amino N-terminal groups in peptide by biotin: application to the detection of specific anti-peptide antibodies by enzyme immunoassays. *J. Immunol. Method* **199:**127-38.

Yamauchi, T., *et al.* (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **423:**462-9.

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermo.com/pierce

Strains Specifications:
BL21(DE3) pET14B-GFP (Amp)
BL21(DE3) pL3SS-mCherry (Cam) incubated on Thursday
Freeze in fridge on Friday over the weekend

For each:

1. Pick 5-10 colonies and inoculate into 10ml LB On Monday
2. Incubate overnight at 37˚C at 160rpm On Monday
3. Prepare 50mL LB with according antibiotics in 250mL baffled shaking flasks On Tuesday
4. Inoculate 500µL of overnight cultures into the flask and incubate at 37˚C at 160rpm On Tuesday
5. Induce with IPTG when OD_{600} =1 On Tuesday
6. Put cultures at room temperature overnight shaking at 160 rpm. On Wednesday

Start Biotinylation experiment