Matchmaker™ Chemiluminescent Co-IP System User Manual

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I. Introduction

The Matchmaker Chemiluminescent Co-IP System (Figure 1) is a convenient coimmunoprecipitation method that utilizes a fluorescent AcGFP1 tag and the enzymatic ProLabel™ reporter for chemiluminescent detection of physical interactions between proteins that are expressed and posttranslationally modified in mammalian cells (1). This system can be used to confirm interactions between bait and prey proteins identified through yeast two-hybrid screening within mammalian cells, without the need for radioisotopes, SDS-PAGE, fluorographic enhancement, or even Western blotting.

The new system is made possible by coupling Clontech's Living Colors® and ProLabel technologies to permit nonradioactive, bright fluorescent monitoring of bait and sensitive, chemiluminescent detection of prey expression (2, 3). AcGFP1 serves as a tag against which anti-AcGFP1 antibody can be used for immunoprecipitation, while the ProLabel enzyme complementation assay offers a quantitative measure of physical interactions between bait and prey proteins that have been expressed and posttranslationally modified in a mammalian system.

The Matchmaker Chemiluminescent Co-IP System consists of the Matchmaker Chemiluminescent Co-IP Vector Set and the Matchmaker Chemiluminescent Co-IP Assay Kit. The vector set contains expression vectors and universal primers for generating in-frame tagged fusion proteins using Clontech's versatile In-Fusion® technology (4), while the assay kit provides a simplified coimmunoprecipitation assay with specialized buffers and a universal primary anti-AcGFP1 antibody for immunoprecipitating interacting complexes.

The Matchmaker™ Chemiluminescent Co-IP System is not only an efficient and effective validation approach, but it is also a safe and sensitive method for verifying direct physical interactions between proteins with proper posttranslational modifications. The system combines the benefits offered by both traditional coimmunoprecipitation assays and mammalian two-hybrid assays, without their limitations and drawbacks, to provide a single assay sufficient for follow-up analysis of yeast two-hybrid screening results.

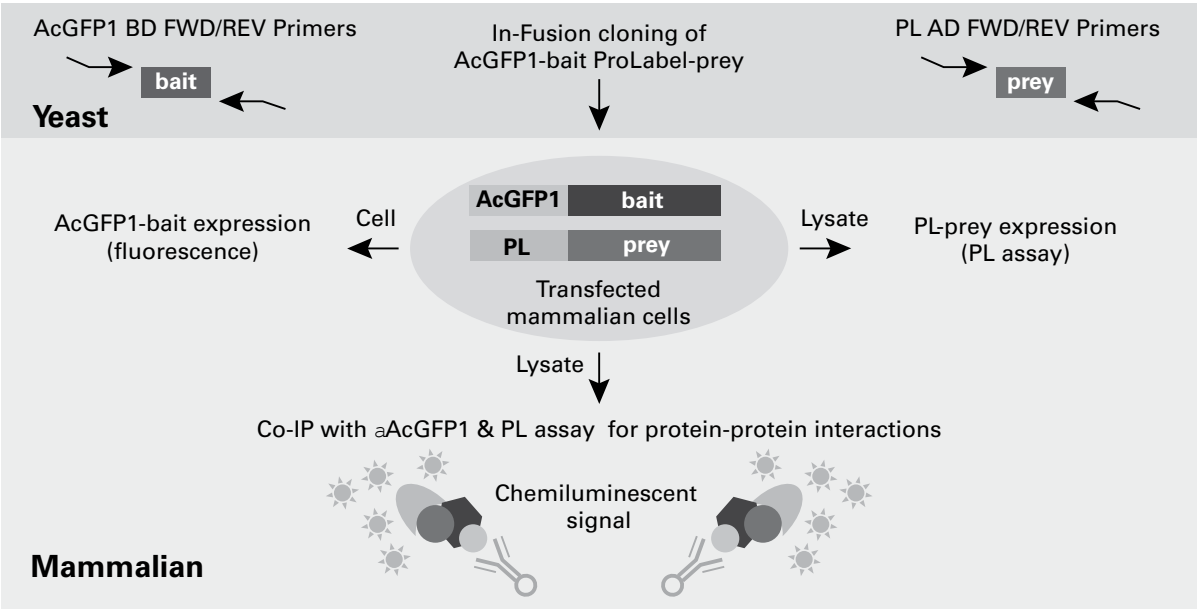

Figure 1.The Matchmaker Chemiluminescent Co-IP System. Our simple and efficient In-Fusion cloning technology is used to transfer bait and prey sequences derived from yeast two-hybrid screening into mammalian expression vectors conferring respective AcGFP1 or ProLabel (PL) tags.The vectors are supplied with AcGFP1 BD FWD/REV (forward/reverse) and PL AD FWD/REV primers to facilitate In-Fusion PCR cloning.

II. List of Components

Matchmaker Chemiluminescent Co-IP Vector Set

Store all components at –20°C. Refer to Appendix C for maps and detailed descriptions of the cloning and control vectors.

- **20 µl pAcGFP1-C Cloning Vector (500 ng/µl)**

  4.7 kb cloning vector used to express an N-terminal AcGFP1-bait fusion in mammalian cells for Co-IP experiments.

- **20 µl pProLabel-C Cloning Vector (500 ng/µl)**

  4.1 kb cloning vector used to express an N-terminal ProLabel-prey fusion in mammalian cells for Co-IP experiments.

- **20 µl pAcGFP1-Lam Negative Control Bait Vector (500 ng/µl)**

  5.6 kb negative control bait vector used to express an N-terminal fusion of AcGFP1 and lamin in mammalian cells.

- **20 µl pAcGFP1-p53 Positive Control Bait Vector (500 ng/µl**)

  5.9 kb positive control bait vector used to express an N-terminal fusion of AcGFP1 and p53 in mammalian cells.

- **20 µl pProLabel-T Control Prey Vector (500 ng/µl)**

  6.2 kb control prey vector used to express an N-terminal fusion of the ProLabel tag and the SV40 large T antigen in mammalian cells.

- **40 µl AcGFP1 BD FWD PCR Primer (10 µM)**

  5'-TAAGGCCTCTGTCGAC**GCCGCCGAGGAGCAGAAGCTGATCTCAGAG**

  Forward PCR primer for amplification of any bait sequence from Clontech's Gal4 BD-based yeast two-hybrid pGBKT7-bait vector for In-Fusion directional PCR cloning into the pAcGFP1-C (SalI/HindIII) vector to yield an in-frame N-terminal fusion of AcGFP1 and the bait sequence.

- **40 µl AcGFP1 BD REV PCR Primer (10 µM)**

  5'-CAGAATTCGCAAGCTT**GTCACTTTAAAATTTGTATACAC**

  Reverse PCR primer for amplification of any bait sequence from Clontech's Gal4 BD-based yeast two-hybrid pGBKT7-bait vector for In-Fusion directional PCR cloning into the pAcGFP1-C (SalI/HindIII) vector to yield an in-frame N-terminal fusion of AcGFP1 and the bait sequence.

- **40 µl PL AD FWD PCR Primer (10 µM)**

  5'-GAATTCTGCAGTCGAC**GCCGCCGAGTACCCATACGACGTACCAGAT**

  Forward PCR primer for amplification of any prey sequence from Clontech's Gal4 AD-based yeast two-hybrid pGADT7-prey vector for In-Fusion directional PCR cloning into the pProLabel-C (SalI/BamHI) vector to yield an in-frame N-terminal fusion of ProLabel and the prey sequence.

- **40 µl PL AD REV PCR Primer (10 µM)**

  5'-TAGATCCGGTGGATCC**AACTTGCGGGGTTTTTCAGTATCTACGATT**

  Reverse PCR primer for amplification of any prey sequence from Clontech's Gal4 AD-based yeast two-hybrid pGADT7-prey vector for In-Fusion directional PCR cloning into the pProLabel-C (SalI/BamHI) vector to yield an in-frame N-terminal fusion of ProLabel and the prey sequence.

II. List of Components, *continued*

Matchmaker Chemiluminescent Co-IP Assay Kit

Store the Polyclonal αAcGFP1 Antibody at –20°C and all other components at 4°C

- 100 ml Cell Lysis Buffer (1X)
- 24 µl Polyclonal anti-AcGFP1 Antibody*
- 600 µl Protein G Plus/Protein A Agarose Beads (50% slurry)
- 100 ml Wash Buffer 1
- 100 ml Wash Buffer 2

III. Additional Materials Required

PCR Cloning

- Advantage® HD Polymerase (Cat. No. 639241)
- Restriction enzymes (SalI, HindIII, BamHI, DpnI)
- NucleoSpin® Extract II Kit (Cat. No. 740609.250)
- In-Fusion™ Dry Down PCR Cloning Kit (Cat. Nos. 639602 & 639604)
- Fusion Blue™ Competent Cells (Cat. Nos. 636700 & 636758)

Transfection

- Transfection reagents
- Culture medium (DMEM, FBS, Sodium Pyruvate)
- PBS
- Trypsin

Coimmunoprecipitation

- 100X HALT Protease Inhibitor Cocktail Kit (Pierce; Cat. No. 78410)
- 100 mM PMSF
- ProLabel™ Detection Kit II (Cat. No. 631629)
- BCA Protein Assay Kit (Pierce; Cat. Nos. 23225 & 23227)
- Monolight 96-well reader & a black 96-well plate with clear, flat bottom
- Barnstead/Thermolyne LABQUAKE Shaker/Rotisserie

IV. In-Fusion™ PCR Cloning of AcGFP1-bait and ProLabel-prey

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. General Considerations

The universal In-Fusion primers provided in the kit are specifically designed to facilitate the directional PCR cloning of inserts from the yeast two-hybrid vectors (listed below) into the specified sites in the pAcGFP1-C and pProLabel-C vectors to generate in-frame AcGFP1-bait and ProLabel-prey fusions.

AcGFP BD FWD/REV: any bait sequences from the pGBKT7 or pLP-GBKT7 for in-frame In-Fusion PCR cloning into the pAcGFP1-C vector linearized with SalI/HindIII

PL AD FWD/REV: any prey sequences from the pGADT7, pGADT7-Rec, pGADT7-Rec2, pLP-GADT7 for in-frame In-Fusion PCR cloning into the pProLabel-C vector linearized with SalI/BamHI

Please note that these primer sets may share sequence homology with other GAL4-based DNA-BD and AD vectors constructs in addition to those listed here. Please check the boldfaced, underlined portions of the primer sequences against your vector of choice to determine if the primers will anneal in the correct orientations and in-frame positions for use in this In-Fusion PCR cloning application. Additionally, restriction sites other than the ones listed above can be used for inserting the bait and prey sequences; however, different In-Fusion primer designs are necessary for the cloning as well as for generating in-frame fusions.

B. Plasmid DNA Templates and DNA Polymerase

We recommend the use of Advantage HD DNA polymerase for all your PCR amplification and cloning as this enzyme is highly regarded for its high fidelity, superior specificity and robust yield. Moreover, Advantage HD has been tested extensively with our Matchmaker Chemiluminescent Co-IP system and consistently has given us excellent results. Purified plasmid DNA from *E. coli,* such as miniprep, midiprep, and maxiprep DNA are all suitable templates for PCR amplification when using Advantage HD. We do not recommend using bacterial colonies harboring your plasmid DNA as a template for PCR amplification. When using Advantage HD DNA polymerase to amplify your bait or prey sequence for In-Fusion PCR cloning, it is important to not exceed the amount of template recommended for the PCR reaction. This is to ensure a clean PCR product that will not yield unwanted background in the cloning step.

C. Restriction Digestion of Cloning Vectors

In-Fusion PCR cloning is extremely efficient provided that the vector backbone is completely linearized at the cloning sites, preferably by two different restriction enzymes.

Consequently, pAcGFP1-C should be digested well with SalI/HindIII and pProLabel-C with SalI/BamHI in a sequential manner to prevent generating background from circular and/or single-cut vector. If you were planning to clone the bait and prey sequences via conventional methods, we would still recommend that you use these same sites when at all possible, as they tend to yield cleaner cuts and less uncut/single-cut background. Just make certain that your cloning design will give you an in-frame fusion.

D. Amplification of Bait with Universal In-Fusion Primers

- 1 µl pGBKT7-bait template (1ng/ul) or water for negative control
- 10 µl 5X Advantage HD PCR buffer
- 4 µl dNTP mix (2.5 mM each)
- 1 µl AcGFP1 BD FWD (10uM)
- 1 µl AcGFP1 BD REV (10uM)
- 0.5 µl Advantage HD polymerase
- 32.5 µl water

50 µl reaction (total volume)

IV. In-Fusion™ PCR Cloning of AcGFP1-bait and ProLabel-prey, *continued*

E. Amplification of Prey with Universal In-Fusion Primers

- 1 µl pGADT7-prey template (1ng/ul) or water for negative control
- 10 µl 5X Advantage HD PCR buffer
- 4 µl dNTP mix (2.5 mM each)
- 1 µl PL AD FWD (10 µM)
- 1 µl PL AD REV (10 µM)
- 0.5 µl Advantage HD Polymerase
- 32.5 µl water

50 µl reaction (total volume)

F. Thermocycler program

This is the initial thermocycler program we recommend for use with the primers provided in the kit.

98°C	5 min
30X	
98°C	15 sec
55°C	15 sec
72°C	3 min*
4°C	soak

* **NOTE:** As a general rule of thumb, the extension time should be 1 min/kb, but 3 minutes will work for the majority of the cDNAs in the library. If you know that your cDNA is longer than 3 kb, then set the time accordingly.

The PCR products can be stored at –20°C after the amplification or analyzed by running 5 µl of each product on a 1% agarose/TAE/EtBr gel alongside a standard, such as 1 kb Ladder, to assess the yield and specificity of the product. There should only be one major band.

Any bait sequence PCR-amplified from the pGBKT7-based vector needs to be digested with DpnI to reduce uncut vector background that would result from PCR template containing the same kanamycin resistance antibiotic marker as the cloning vector, pAcGFP1-C. Sequences amplified from pGADT7-based vector need not be digested with DpnI since this template has an Amp^R marker, which is different from the Kan^R marker present in the pProLabel-C vector, and thus will not be contributing to any background, as it will be selected against on a kanamycin-containing plate. To treat the PCR product with DpnI, simply add 1 µl of DpnI to the remaining 45 µl of PCR product and incubate for 60 minutes at 37°C. If you are not proceeding with the In-Fusion cloning, store the DpnI-treated PCR product at –20°C.

****GOOD STOPPING POINT****

IV. In-Fusion™ PCR Cloning of AcGFP1-bait and ProLabel-prey, *continued*

G. In-Fusion Cloning

We recommend that all the linearized cloning vectors and PCR products be purified using our NucleoSpin Extraction columns (according to manufacturer's instructions) to remove the enzymes and free dNTPs, prior to using them in the In-Fusion cloning. Determine the concentration of your purified PCR-amplified bait and prey sequence as well as your linearized pAcGFP1-C (SalI/HindII) and pProLabel-C (SalI/BamHI). Set up the In-Fusion reactions as follows:

1A. Vector control

- 100 ng pAcGFP1-C (SalI/HindIII)
- water

10 µl total volume

1B. Vector + bait

- 100ng pAcGFP1-C (SalI/HindIII)
- 50–200 ng bait *
- water

10 µl total volume

2A. Vector control

- 100 ng pProLabel (SalI/BamHI)
- water

10 µl total volume

2B. Vector + prey

- 100 ng pProLabel-C (SalI/BamHI)
- 50–200 ng prey *
- water

10 µl total volume

* **NOTE:** Use the appropriate amount corresponding to the length of PCR product:

25 ng	0.5 kb PCR product
50 ng	1 kb PCR product
100 ng	2 kb PCR product
150 ng	2.5 kb PCR product
200 ng	3.0 kb PCR product

Transfer the liquid content of each sample to a tube of In-Fusion Dry-Down Mix. Pipet gently to mix and dissolve the dry-down "cake" and allow the reaction to incubate for 15 min at 37°C and 15 min at 50°C in a thermocyler with a heated lid. After the incubation, promptly remove the samples and place on ice if proceeding with the transformation. Otherwise, store the reactions at –20°C until use.

****GOOD STOPPING POINT****

IV. In-Fusion™ PCR Cloning of AcGFP1-bait and ProLabel-prey, *continued*

H. Transformation

1. Dilute the In-Fusion reaction mixture with 40 ul TE buffer [pH 8.0], mix well, and place on ice.
2. Transform competent cells with the diluted In-Fusion reaction as follows:
   a. Using Fusion-Blue™ Competent Cells:

   - For each transformation, thaw one vial of frozen Fusion-Blue Competent cells on ice. **NOTE:** It is a good idea to also set up a control transformation using circular DNA such as pUC19 to assess the transformation efficiency.
   - Tap tubes gently to ensure that the cells are suspended.
   - Add 2.5 µl of the diluted reaction mixture or 1 ng of control circular DNA to the cells. Mix gently to ensure even distribution of the DNA solution.

     NOTE: Do not add more than 5 µl of the diluted In-Fusion reaction to 50 µl of competent cells.
   - Leave the tubes on ice for 30 minutes.
   - Heat shock the cells in a 42°C water bath for 45 sec, and then place them directly on ice for 2 min.

   b. If using other competent cells with In-Fusion™ Kits, make sure that the efficiency of the competent cells used for the transformation is > 1 x10^8 cfu/ug. Follow the transformation protocol provided by the manufacturer and proceed to Step 3.

      NOTE: Do not add more than 5 ul of the diluted In-Fusion reaction to 50 µl of competent cells.
3. After heat shocking, add 950µl of SOC medium to the cell. Incubate at 37°C for 60 min while shaking at 250 rpm.
4. For each transformation, plate 50–100 µl of the culture onto an LB/Kan (50 µg/ml) agar plate. Pellet the cells from the remaining culture, discard the medium except for about 50–100 µl and spread the entire remaining transformed cells onto another LB/Kan (50 µg/ml) agar plate. Incubate all plates at 37°C overnight.
5. The next day, pick colonies from each experimental plate and isolate plasmid DNA using a standard method of choice.
6. Assess for positive clones by either colony PCR screening or restriction digest of miniprep DNA. If desired, sequencing can be performed at the cloning junctions to further verify that the clones contain the bait and prey sequences and that that the fusions are all in-frame.
7. Pick one positive clone for the bait and one for the prey to prepare a large-scale DNA preparation for use in the transfection and Co-IP experiments. DNA preparations can be stored at –20°C until used for transfection.

****GOOD STOPPING POINT****

I. Verification of AcGFP1-Bait and ProLabel-Prey Expression

It is important that mammalian expressions of the bait and prey be verified prior to performing your Co-IP experiment. This step is easily done now that your bait and prey are fused with AcGFP1 and ProLabel respectively.

1. Using a cell line* and a transfection reagent of choice, set-up 2 small-scale (12-or 24-well plate) cotransfections according to the procedures recommended by the transfection reagent's manufacturer.

   a. pAcGFP1-C and pProLabel-C

   b. pAcGFP1-bait and pProLabel-prey

   *We typically use CalPhos Mammalian Transfection Kit (Clontech Cat. No. 631312) and HEK 293 cells as this combination gives consistently high transfection efficiency.

IV. In-Fusion™ PCR Cloning of AcGFP1-bait and ProLabel-prey, *continued*

2. 48-hr posttransfection, observe the transfected cells with fluorescent microscopy using a filter for detection of AcGFP1. Expression of the AcGFP1-bait fusion can be monitored and confirmed by the presence of green fluorescence in cells transfected with pAcGFP1-bait and pProLabel-prey. Keep in mind that cells transfected with pAcGFP1-C and pProLabel-C will also contain green fluorescence, however, the expression level, pattern and brightness of your AcGFP1-bait fusion may very well be different from those exhibited by AcGFP1 alone. If necessary, FACS or Western analysis can be used to assess whether your AcGFP1-bait is being expressed.

3. To assay for your ProLabel-prey expression, you will need the ProLabel Detection Kit II. Please refer to Appendix A for preparation of the lysis/complementation buffer and substrate mix for the ProLabel enzyme complementation assay.

   a. Remove the medium from the well and wash the cells with 500 µl of PBS.

   b. Aspirate off the PBS and keep the plate containing the cells on ice.

   c. To each well, add 200 µl of the lysis/complementation assay buffer.

   d. Pipet up and down several times to dislodge and lyse the adherent cells.

   e. For each sample being assayed, transfer 80 µl of the lysate to a 96-well plate with clear bottom and black side.

   f. To each 80 µl of lysate being assayed, add 30 µl of the substrate mix.

   g. Gently pipet up and down twice to mix the content.

   h. Incubate the plate at room temperature for 15 min and up to 1 hr.

   i. Using a luminometer, record ProLabel activity every 15 min during this time interval.

4. Different ProLabel fusions will yield different levels of ProLabel activity. However, if your ProLabel-prey fusion is being expressed in cells cotransfected with pAcGFP1-bait and pProLabel-prey, the ProLabel activity detected should be significantly higher that the one observed in the negative control—i.e., cells cotranfected with pAcGFP1-C and pProLabel-C as this should not yield any significant ProLabel activity.

J. Cotransfection of Mammalian Cells for Co-IP Experiments

We typically use calcium phosphate to cotransfect HEK 293 cells as combination consistently gives high transfection efficiency. If you wish to use a different transfection reagent and/or cell line, just make certain that the selected transfection reagent is capable of giving high transfection efficiency in your particular cell line.

1. For each cotransfection, you will need one 60-mm plate.
2. One day before the transfection seed cells onto 60 mm plates at a density such that the next day the cells will be roughly 50-75% confluent. For HEK 293 cells, this means that approximately 1 x 10^6 cells are seeded onto each 60-mm plate.
3. Set up the control and experimental cotransfections, using 4 µg of DNA for each construct.

   • Negative control: pAcGFP1-Lam (4 µg) + pProLabel-T (4 µg)

   • Positive control: pAcGFP1-p53 (4 µg) + pProLabel-T (4 µg)

   • Experimental controls*: pAcGFP1-neg (4 µg) + pProLabel-prey (4 µg) OR

   pAcGFP1-bait (4 µg) + pProLabel-neg (4 µg)

   • Experimental sample: pAcGFP1-bait (4 µg) + pProLabel-prey (4 µg)

   ***NOTE:** The empty pAcGFP1-C or pProLabel-C construct can be used if a negative bait/prey is not available for use as a negative control for your experimental sample.

4. Transfect according to the protocols recommended by the reagent's manufacturer.

IV. In-Fusion™ PCR Cloning of AcGFP1-bait and ProLabel-prey, *continued*

5. Between 24–48 hours posttransfection, examine the cells under the fluorescent microscope to see if the fluorescence from the AcGFP1 fusion can be observed. The percentage of cells positive for green fluorescence can be used as a measure of your transfection efficiency as well as a way to monitor the expression of your bait.
6. 48-hr post-transfection, wash the cells in each plate with 2x 5 ml of PBS (prewarmed to 37°C).
7. Aspirate off the PBS and trypsinize the cells in each 60-mm plate with 2mL of trypsin (prewarmed to 37°C for 3–5 minutes. Dislodge any remaining adherent cells by either pipetting gently or by knocking the plate sideways against the palm of your hand.
8. Add 5 ml of DMEM + 10% FBS (prewarmed to 37°C) to stop the trysinization process and use a pipet to transfer the cells from each plate to a clean 15 ml conical tube.
9. Pellet the cells at 500 x g for 5 minutes and remove the supernatant, taking care not to disturb the cell pellets.
10. Wash the cell pellets two more times, each time with 10 mL of ice cold PBS.
11. Remove as much of the PBS as possible from each tube without disturbing the pellet.
12. At this stage, the cell pellets can be used immediately to prepare cell lysate for the Co-IP assay or the pellets can be stored at -70oC until use. If you intend to lyse the cell pellets at this stage, then the pellets must be kept on ice at all times.

V. Chemiluminescent Co-IP Assay Protocol

A. Preparation of Cell Lysates

1. Place the tubes containing the cell pellets on ice to thaw.
2. Determine the total volume of Cell Lysis Buffer required and transfer that amount to a clean 15 ml conical tube.

   You will need to prepare 1 ml of fresh Cell Lysis Buffer with 1X PMSF and 1X protease inhibitor cocktail for each cell pellet sample—500 µl for the cell lysis and the remaining 500 µl for the dilution of the lysate for the coimmunoprecipitation step.
3. Add a sufficient amount of the 100X PMSF and 100X protease inhibitor cocktail to the Cell Lysis Buffer to give the Cell Lysis Buffer a final concentration of 1X PMSF/1X protease inhibitor cocktail.

   NOTE: PMSF is labile in aqueous solution, so add it to the Cell Lysis Buffer just before using.
4. Cap and mix thoroughly by inversion several times and then place the Cell Lysis Buffer/1X PMSF/1X protease inhibitor cocktail on ice to chill.
5. Transfer 500 µl of the cold Cell Lysis Buffer/1X PMSF/1X protease inhibitor cocktail to cell pellet and pipet up and down to disburse the cells.
6. Transfer the entire volume of each crude lysate sample to a clean 1.5 ml microcentrifuge tube and place the tube on ice for 30 min, with brief (5–10 sec) vortexing at 10 min intervals.
7. After the 30 min incubation on ice, pellet the cellular debris by centrifugation at 10K x g for 20 min at 4°C. To prevent degradation of proteins, it is important to cool the microcentrifuge down to 4°C prior to placing the lysate samples into it.
8. Transfer the precleared lysate (supernatant) to another clean 1.5 ml microcentrifuge tube and immediately place it on ice.
9. Determine the total protein concentration in each sample by BCA (see Section III) and record.
10. Based on the protein concentration determined by BCA, transfer 250 µg of each lysate to a clean 1.5 ml microcentrifuge tube and bring the volume of the lysate to a total of 500 µl using the remaining cold Cell Lysis Buffer/1X PMSF/1X protease inhibitor cocktail.
11. Place the tubes containing the diluted lysates on ice if you are planning on proceeding with the coimmunoprecipitation. Otherwise, store both the concentrated and diluted lysates at –70°C until use.

B. Washing of the Protein G Plus/A Agarose Beads

1. Using a P1000 pipetman, gently pipet the Protein G Plus/Protein A agarose beads up and down several times to obtain an even suspension of the beads.
2. Place a clean 1.5 ml microcentrifuge tube for each Co-IP sample on ice.
3. Transfer 25 µl of the agarose bead suspension to each 1.5 ml microcentrifuge tube.
4. Wash the beads twice, each time with 500 µl of cold lysis buffer (no protease inhibitor and no PMSF) by inverting the tube gently 3-4X to resuspend the beads and then pelleting the beads at 10K x g for 30 sec at 4°C.
5. Remove as much of the lysis buffer as possible without disturbing or removing the agarose beads.
6. To the washed beads, add 25 µl of cold lysis buffer (no protease inhibitor/no PMSF) to form a suspension again and place on ice until ready to use, which should be the same day that it is washed.

V. Chemiluminescent Co-IP Assay Protocol, *continued*

C. Coimmunoprecipitation

1. To each 500 µl diluted lysate sample, add 1 µl of the anti-AcGFP polyclonal antibody. Cap the tubes tightly and place them on a rotator at 4°C for 1–2 hr, making sure that the sample is gently rotating during the entire antibody incubation.
2. After the antibody incubation, transfer the entire volume of each sample to the tube containing the 25 µl of washed agarose beads (see Section B) and allow the sample to rock or rotate gently O/N at 4°C.
3. Next morning, pellet the beads GENTLY at 4°C, 5000 x g for 10 sec.
4. Discard the supernatant without pipeting off the beads as these contain the protein complexes to assayed in the ProLabel assay.
5. Wash the beads 5X 500 µl with Wash Buffer 1, each time gently adding the buffer to the tube containing the beads, capping the tube, inverting it several times to gently resuspend the beads into the wash buffer, pelleting the beads at 4°C, 5000 x g for 10 sec, and discarding the wash buffer (supernatant).
6. Wash the beads in 4X 500 µl with Wash Buffer 2, as in step 5, but DO NOT discard the final wash with Wash Buffer 2
7. Place the tubes containing the beads on ice.

D. ProLabel Detection of Protein-Protein Interactions

1. Prepare the lysis/complementation buffer and substrate mix as outlined in Appendix A**.**
2. Remove and discard as much of the final Wash Buffer 2 as possible from the tubes without disturbing the beads (from Step 7 above).
3. Resuspend each sample of beads with 80 µl of the lysis/complementation buffer by gently pipeting up and down to prevent bubbles and then transferring the entire content (beads and buffer) to a well in a 96-well plate (make sure the plate is black with a clear flat bottom).
4. To each well, add 30 µl of substrate mix and read the ProLabel activity from the samples using the Monolight 96-well reader at 0, 10, 15, 20, 25, 30, and 45 min after addition of substrate.
5. Plot the ProLabel readings as a function of time to qualitatively assess that at least two of the time points between 10’ and 30’ are within the linear range of the ProLabel enzymatic activity.
6. Pick a time point in the linear range that has the highest readings to calculate fold of induction.

VI. Troubleshooting Guide

If you do not obtain the expected results, use the following guide to troubleshoot your experiment. To confirm that your kit is working properly, perform the control reactions (Section IV.J., Step 3)

TABLE I. TROUBLESHOOTING GUIDE FOR IN-FUSION™ CLONING

Description of Problem	Explanation	Solution
No PCR product observed	PCR component missing or degraded	Use a checklist when assembling reaction. Always perfom a positive control to ensure that each component is functional. If the positive control does not work, repeat the positive control only. If the positive control still does not work, repeat again, replacing individual components to identify the faulty reagent.
	Poor template quality	Check template integrity by electrophoresis on a standard TAE- or TBE-agarose gel. If necessary, repurify your template using methods that minimize shearing and nicking. Be sure that you are not using a bacterial colony as the template, as such cellular debris will inhibit the activity of Advantage HD DNA Polymerase.
	Difficult target	Some targets are inherently difficult to amplify. In most cases, this is due to unusually high GC-content and/or secondary struction. In some cases, the addition of DMSO to 2–5% may help.
Multiple smears in PCR product	Contamination	See PCR contamination, next page.
	Too much template	The amount of plasmid template recommended when using Advantage HD DNA polymerase is 10 pg ~ 1 ng per 50 µl reaction. If too much template is added, additional bands or a smear may be observed. This can be improved by reducing the amount of template used in the PCR reaction.
	Too many cycles	Reducing the cycle number by 3–5 may eliminate nonspecific bands.
	Multiple PCR products	If your PCR product is not a single distinct band, then it may be necessary to gel purify the PCR product to ensure cloning of the correct insert. See PCR contamination, next page.

VI. Troubleshooting Guide, *continued*

TABLE I. TROUBLESHOOTING GUIDE FOR IN-FUSION™ CLONING

Description of Problem	Explanation	Solution
PCR contamination		Contamination most often results in extra bands or smearing. It is important to include a water control (i.e., a control using ddH_2O as the template") in every PCR experiment to determine if the PCR reagents, pipettors, or PCR reaction tubes are contaminated with previously amplified targets. If possible, set up PCR reactions and perform post-PCR analysis in separate laboratory areas with separate sets of pipettors. It is advisable to use one of the commercially available aerosol-free tips. Laboratory benches and pipettor shafts can be decontaminated by depurination. Wipe surfaces with 1N HCl followed by 1N NaOH. Then neutralize with a neutral buffer (e.g., Tris or PBS) and rinse with ddH_2O. There is an enzymatic method for destroying PCR product carryover (Longo et al., 1990). It involves incorporation of dUTP into the PCR products and susequent hydrolysis with uracil-B-glycosylase (UNG).

VI. Troubleshooting Guide, *continued*

TABLE I. TROUBLESHOOTING GUIDE FOR IN-FUSION™ CLONING

Description of Problem	Explanation	Solution
Low cloning efficiency	High vector background	This can be the result of one or a combintion of two factors–incomplete digestion of the pAcGFP1-C cloning vector with SalI/HindIII or of pProLabel-C with SalI/BamHI, or by forgetting to treat the PCR product with DFpnI. If the cloning vector is not digested completely, the remaining circular or religated single-cut vector can contribute to the background in the cloning. Likewise, if you forget to treat your bait PCR product with DpnI to remove the Kan^R circular template used in the PCR reaction, this circular template will also result in high vector background. It is also important not to unnecessarily use more template in the PCR reaction than is suggested, as this will require a lengthier period of DpnI treatment than the time recommended in this manual,
	Few or no recombinant clones	If you are using the provided Universal In-Fusion Primers for the amplification and In-Fusion PCR cloning of your bait and prey but are unable to obtain recombinant clones despite low vector backgrounds, please check to make sure that the cloning vector is digested with the correct restriction enzymes. Also check to make sure that the In-Fusion reaction has been diluted properly prior to transforming Fusion Blue cells. Verify the efficiency of the competent cells as well as the proper antibiotic used in selecting the recombinant clones. If you are designing your own In-Fusion primers, verify that the regions of homology are as suggested in the In-Fusion PCR Cloning Manual
Transfection efficiency		The efficiency of transfection for different cell lines may vary by several orders of magnitude. A method that works well for one host cell line may be inferior to another. Therefore, when working with a cell line for the first time, you may want to compare the efficiencies of several transfection protocols. This can easily be done by cotransfecting your cells of interest with the pAcGFP1-C and the pProLabel-C constructs and comparing the number of cells that are positive for fluorescence against the total number of cells in the same field under phase contrast. After a method of transfection is chosen, it may be necessary to optimize parameters such as cell density, amount and purity of the DNA, media conditions, and transfection time. Once optimized, these parameters should be kept constant to obtain reproducible results. With each method, AcGFP1 fluorescence may be detected 24–72 hours after transfection, depending on the host cell line used.
Low expression of bait/prey		If there is no expression of bait/prey observed, it is a good idea to verify by sequencing that the bait and prey sequences are in-frame with the AcGFP1 and ProLabel tags respectively. However, if the fusions are in-frame but expression is low yet detectable via fluorescence or ProLabel assay, the low levels of expression may just represent natural steady state levels of the particular bait and/or prey and should not affect the coimmunoprecipitation assay, as the polyclonal anti-AcGFP1 antibody is very specific and the ProLabel assay is a highly sensitive assay—capable of detecting the interaction despite low expression levels.

VI. Troubleshooting Guide, *continued*

TABLE I. TROUBLESHOOTING GUIDE FOR IN-FUSION™ CLONING

Description of Problem	Explanation	Solution
Co-IP background	Insufficient washing	High ProLabel signals from the negative control are typically the result of insufficient washing. Try increasing the number of washes as well as the volume of each wash. Some proteins are naturally "sticky" and may require slightly more stringent wash conditions, such as increased salt concentration. You may also try preclearing the lysate with 25 µl of the Protein A/G agarose beads before proceeding with the Co-IP to remove any nonspecific binding to the Protein A/G agarose beads.
	Too much antibody or lysate	Check to make sure that you are using the recommended amounts of antibody and lysate for each Co-IP assay.
Low ProLabel readings, but high fold of induction	Some ProLabel readings are found have slower enzyme kinetics (i.e., the chemiluminescent signal accumulates at a slower rate than for some other ProLabel fusions)	Allow the signal to accumulate and take additional readings at 45 and 60 min.
Low fold of induction	Weak interaction between your bait and prey proteins	Allow the signal to accumulate and take additional readings at 45 and 60 min.
No significant ProLabel activity in the coimmunoprecipitate, but expression of both AcGFP1-bait and ProLabel-prey fusions have been confirmed.	Bait and prey do not physically interact. Pair may possess only genetic interactions or may be the result of a false positive isolated from the yeast two-hybrid screen	Check to make sure that antibody and agarose beads have been added. If the +/- controls are working, but the experimental samples are not giving any signal, then the explanation to the left pertains.

VII. References

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Appendix A: ProLabel Enzyme Complementation Assay

Thaw all of the components from the ProLabel Detection Kit II at room temperature. Once the components are thawed, invert the mix and then place the components on ice.

A. Lysis/Complementation Buffer

Combine 3 volumes of Cell Lysis Buffer* with 1 volume of EA. Mix well and place on ice until use. The volumes can be scaled accordingly depending on how many samples are being assayed. For verification of ProLabel-prey expression, you will need 200 µl per sample. For detection of bait-prey interaction in the coimmunoprecipitate, you will need 80 µl per Co-IP sample. It is a good idea to prepare 10% extra to account for pipetting error.

*__NOTE:__ This is the Cell Lysis Buffer provided in the ProLabel Detection Kit II and not the Cell Lysis Buffer provided in the Matchmaker Chemiluminescent Co-IP Assay. Only the Cell Lysis Buffer provided in the ProLabel Detection Kit should be used here as it has been optimized specifically for ProLabel activity.

B. Preparation of Substrate Mix

For each ProLabel assay, you will need to mix the following:

1.2 µl of Galacton

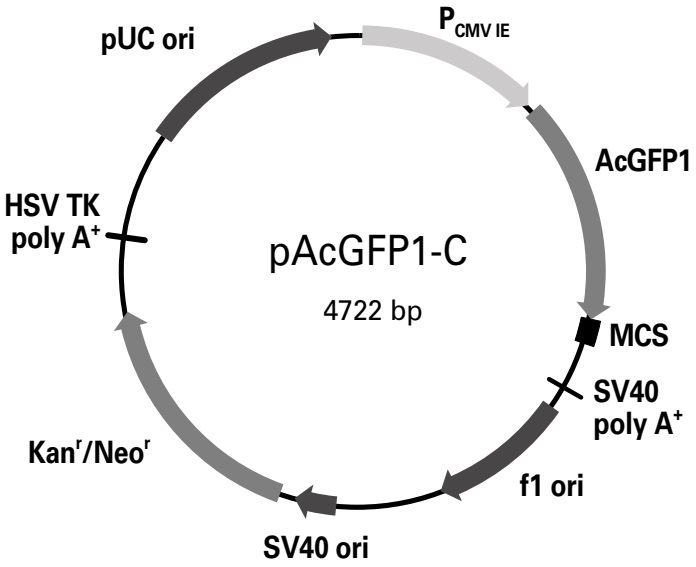
6 µl of Emerald

22.8 µl of Substrate

30 µl total per assay

Again, to scale up, the volume of each component will need to be multiplied by the number of samples that are being assayed. It is a good idea to prepare 10% extra to account for pipeting error.

Appendix B: Plasmid Maps and Multiple Cloning Sites



pAcGFP1-C MCS

```
                                                                MCS
                AcGFP1                                  SalI        BglII       HindIII
                                                                                    EcoRI
  1   ATGGATGAGC TGTACAAGTC CGGACTTAAG GCCTCTGTCG ACCAGATCTC TAAGCTTGCG
      TACCTACTCG ACATGTTCAG GCCTGAATTC CGGAGACAGC TGGTCTAGAG ATTCGAACGC
                MCS
      EcoRI       BamHI           XbaI        BclI
 61   AATTCTGGCG GATCCACCGG ATCTAGATAA CTGATCAT
      TTAAGACCGC CTAGGTGGCC TAGATCTATT GACTAGTA
```

Figure 2. pAcGFP1-C Vector Map and Multiple Cloning Sites

Description. pAcGFP1-C Vector is a mammalian expression vector that encodes a green fluorescent protein (GFP) from *Aequorea coerulescens*. The fluorescent protein coding sequence in this construct has been human-codon-optimized for efficient expression and enhanced brightness. AcGFP1 protein has an excitation maximum at 475 nm and an emission maximum at 505 nm. Sequences flanking AcGFP1 have been converted to a Kozak consensus translation initiation site (1) to further increase translation efficiency in eukaryotic cells. A gene of interest can be added in-frame downstream of the AcGFP1 coding sequence and expressed as a fusion protein to the C-terminus of AcGFP1. SV40 polyadenylation signals downstream of the AcGFP1 gene direct proper processing of the 3′end of the mRNA transcript. In addition, the vector also contains a SV40 origin of replication in mammalian cells expressing the SV40 T-antigen. A neomycin resistance cassette (Neo^r) containing an SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSVTK) gene, allows selection of stable transformants in eukaryotic cells using G418. A bacterial promoter upstream of the gene expresses kanamycin resistance in *E. coli.* pAcGFP-C Vector also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

Appendix B: Plasmid Maps and Multiple Cloning Sites *continued*

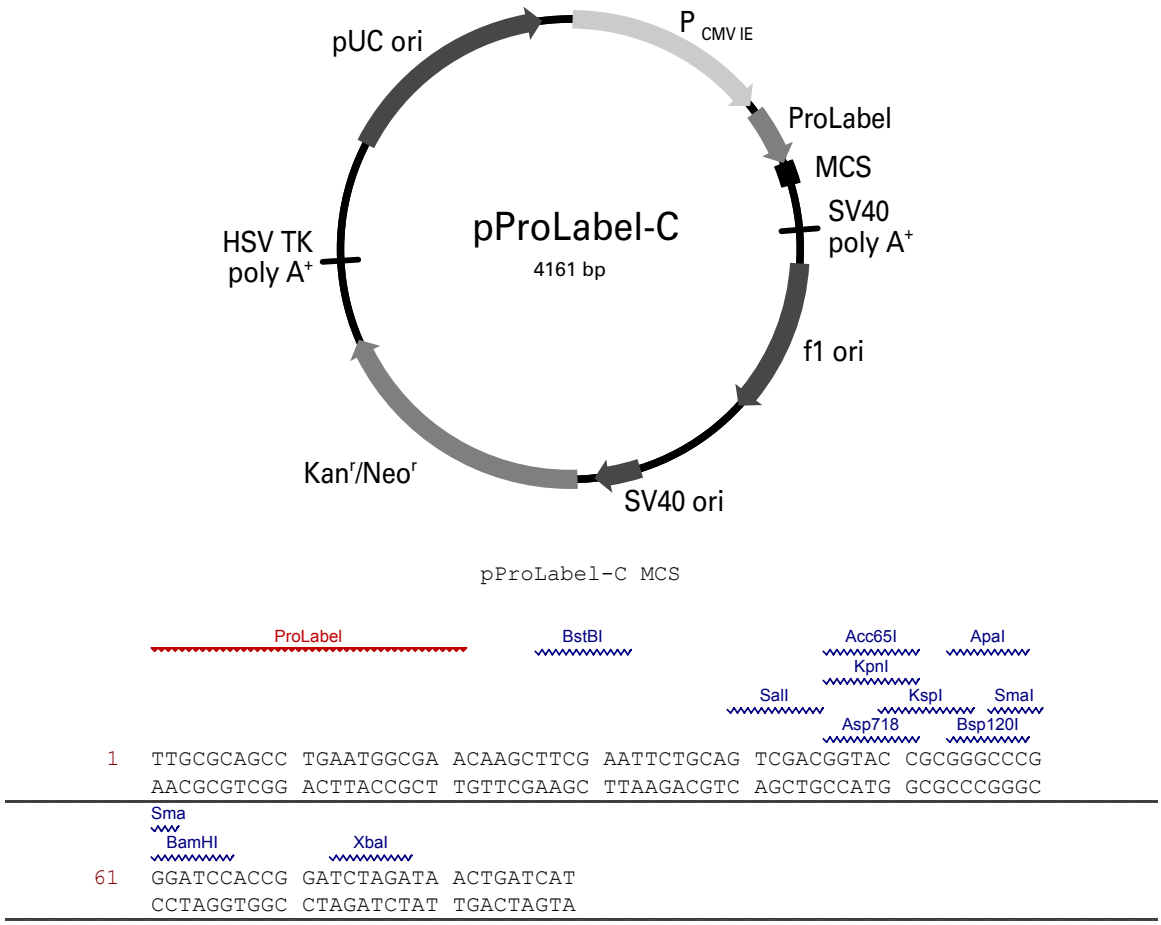


Figure 3. pProLabel-C Vector Map and Multiple Cloning Sites

Description. pProLabel-C Vector is a linearized mammalian expression vector encoding the ProLabel tag (~6 kDa). The coding sequence of the ProLabel tag is comprised of the a fragment of β-galactosidase which, when combined with the Ω fragment of β-galactosidase, produces an active β-galactosidase enzyme.The MCS of pProLabel-C has been linearized with SalI/BamHI and genes cloned into the vector at this site will be expressed as fusions to the C-terminus of the ProLabel tag if they are in the same reading frame as the ProLabel and there are no intervening stop codons. SV40 polyadenylation signals downstream of the ProLabel gene direct proper processing of the 3′ end of mRNA. The vector backbone also contains an SV40 origin for replication in any mammalian cell line that expresses the SV40 T antigen. A neomycin resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene ofTn5, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSV-TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette drives expression of the gene encoding kanamycin resistance in *E. coli.* The pProLabel-C vector backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

Appendix B: Plasmid Maps and Multiple Cloning Sites *continued*

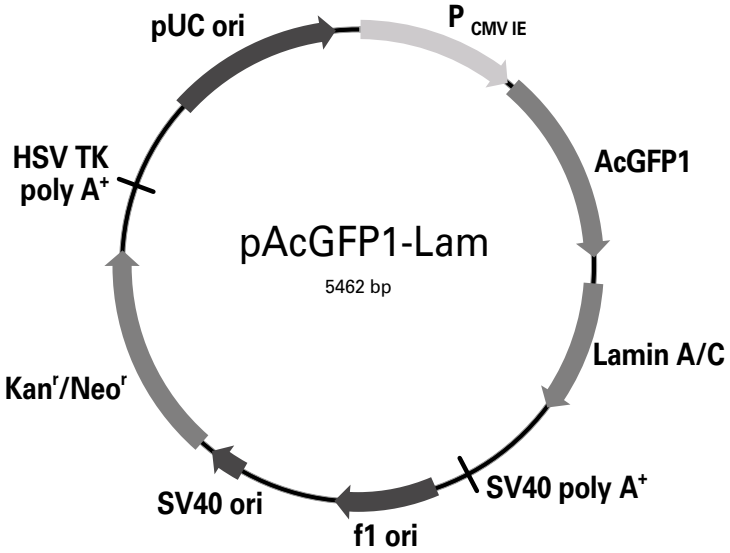


Figure 4. pAcGFP1-Lam Vector Map.

Description. pAcGFP1-Lamin encodes a green fluorescent protein (GFP) from *Aequorea coerulescens* (excitation maximum = 475 nm; emission maximum = 505 nm) and the gene encoding the human nuclear lamin (1). SV40 polyadenylation signals downstream of the AcGFP1-Lamin fusion direct proper processing of the 3'end of the AcGFP1-Lamin mRNA. AcGFP1 contains silent mutations that create an open reading frame comprised almost entirely of optimized human codons. These changes increase the translational efficiency of the AcGFP1 mRNA and consequently the expression of AcGFP1 in mammalian and plant cells. The vector backbone also contains an SV40 origin for replication in any mammalian cell line that expresses the SV40 T-antigen. A neomycin resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSV-TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette drives expression of the gene encoding kanamycin resistance in *E. coli.* The pAcGFP1-Lamin backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

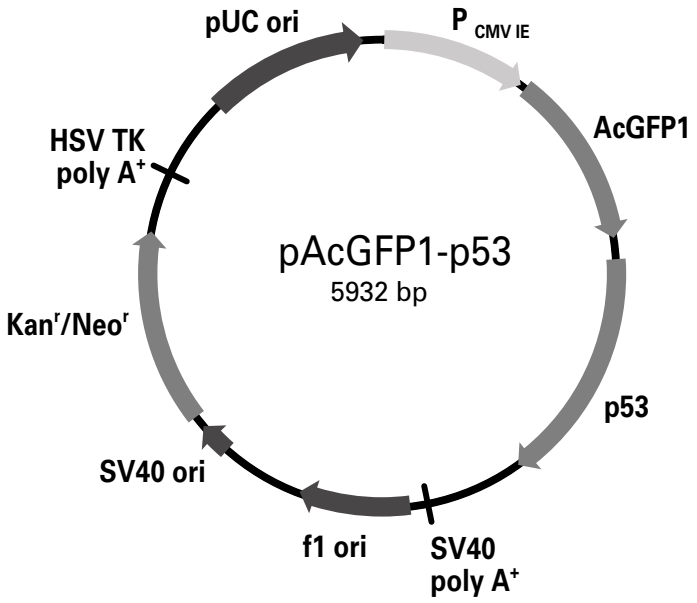


Figure 5. pAcGFP1-p53 Vector Map

Description. pAcGFP1-p53 Vector encodes a green fluorescent protein (GFP) from *Aequorea coerulescens* (excitation maximum = 475 nm; emission maximum = 505 nm) and the gene encoding the human tumor suppressor p53. SV40 polyadenylation signals downstream of the AcGFP1-p53 fusion direct proper processing of the 3′end of the AcGFP1-p53 mRNA. AcGFP1 contains silent mutations that create an open reading frame comprised almost entirely of optimized human codons. These changes increase the translational efficiency of the AcGFP1 mRNA and consequently the expression of AcGFP1 in mammalian and plant cells.The vector backbone also contains an SV40 origin for replication in any mammalian cell line that expresses the SV40T antigen. A neomycin resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene ofTn5, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSV-TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette drives expression of the gene encoding kanamycin resistance in *E. coli*. The pAcGFP1-p53 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

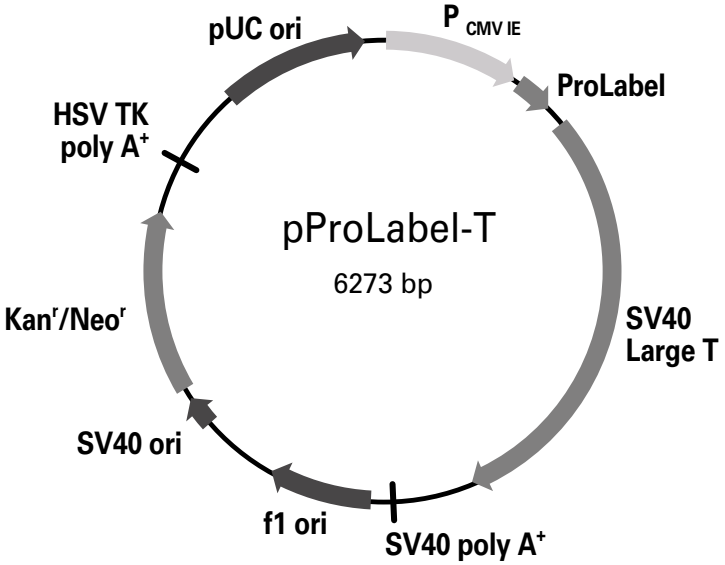


Figure 6. pProLabel-T Vector Map

Description. pProLabel-T Vector is a mammalian expression vector encoding a fusion of the SV40 large T antigen at the C-terminus of the ProLabel tag (~6 kDa). Because ProLabel is the a fragment of the split β-galactosidase enzyme, the ProLabel-T fusion protein alone has no enzymatic activity. However, the ProLabel-T fusion can recombine with the Ω fragment of β-galactosidase to reconstitute an active enzyme. The SV40 polyadenylation signals downstream of the ProLabel-SV40 T gene direct proper processing of the 3'end of mRNA. The vector backbone also contains an SV40 origin for replication in any mammalian cell line that expresses the SV40T antigen. A neomycin resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSV-TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette drives expression of the gene encoding kanamycin resistance in *E. coli*. The pProLabel-C vector backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

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