# Protein-DNA Binding Assay User Manual

Cat. No. 630460
PT3988-1(PR782347)
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# I. Introduction & Protocol Overview

## Chemiluminescent ProLabel™ Detection of Protein-DNA Binding

The **Protein-DNA Binding Assay** (Cat. No. 630460) provides a safe, fast, and sensitive alternative to traditional electromobility shift assays (EMSA) for detection and quantitative characterization of protein-DNA interactions. The binding assay is performed in a 96-well plate, thereby eliminating the need for gel electrophoresis. It also abolishes the need for radioactive labeling of nucleic acids because the assay is reformatted to take advantage of Clontech's sensitive and quantitative ProLabel™ chemiluminescence detection technology. This method consists of fusing a small (~6 kDa) ProLabel tag to your protein of interest. The resulting ProLabel fusion protein is capable of producing a strong chemiluminescent signal via the ProLabel enzyme complementation assay (Figure 1; July 2007, *Clontechniques*). Thus, the ProLabel tag allows direct detection of specific binding between your protein of interest and a dsDNA oligonucleotide, without the need for antibodies or radiolabeling. Moreover, because the ProLabel fusion protein is expressed in mammalian cells, it can acquire biologically relevant posttranslational modifications that may be necessary for functional DNA binding (Tootle *et al.*, 2005).

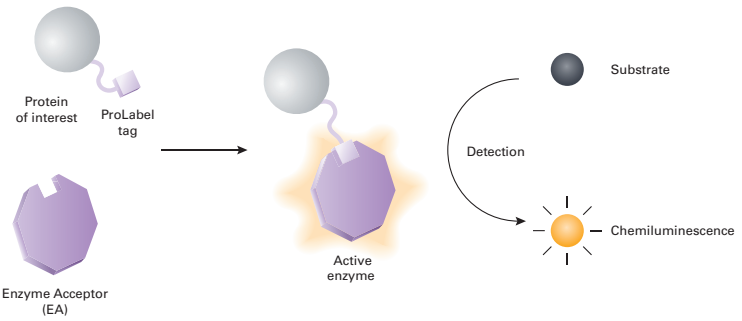



**Figure 1. The ProLabel screening assay.** The ProLabel tag on the fusion protein complements the function of the Enzyme Acceptor. The ProLabel tag and the Enzyme Acceptor combine to form an active enzyme that cleaves the chemiluminescent substrate, and produces a signal that can be detected with any standard luminometer.

## A Complete Assay System for Cloning, Expression & Detecting Protein-DNA Binding

The binding reaction is carried out by incubating a cellular extract containing the ProLabel fusion protein of interest with a biotinylated dsDNA oligonucleotide containing a putative consensus binding sequence for this protein (Figure 2). The biotin moiety on the oligonucleotide permits its subsequent capture on a streptavidin-coated 96-well plate. Then the wells are subjected to a series of wash steps to remove nonspecific binding interactions and minimize background signal. Specific protein-DNA binding interactions are measured using the ProLabel assay.

The Protein-DNA Binding Assay provides the pProLabel-C Vector for cloning and expressing your ProLabel fusion protein of interest in mammalian cells. The kit also includes specially formulated buffers for preparing whole cell extracts and performing the binding assay. A streptavidin-coated 96-well plate for capturing the protein-DNA complexes and the ProLabel Detection Kit II are also included. In addition, the kit provides a control vector containing ProLabel fused to a known DNA binding protein, as well as biotinylated dsDNA oligonucleotides designed to serve as positive and negative DNA-protein binding controls, respectively, for the assay that examines the binding of the p53 fusion protein to its cognate *cis*-acting DNA consensus element. If you are confirming protein-DNA interactions identified using our **Matchmaker™ One-Hybrid Library Construction & Screening Kit** (Cat. No. 630304), you can take advantage of our robust and efficient In-Fusion™ PCR Cloning technology by using the included Universal In-Fusion Cloning primers (October 2007, *Clontechniques*). These primers are designed for direct and efficient directional PCR cloning of putative yeast one-hybrid clones from any of Clontech's pGADT7-based cDNA library vectors into the pProLabel-C Vector.

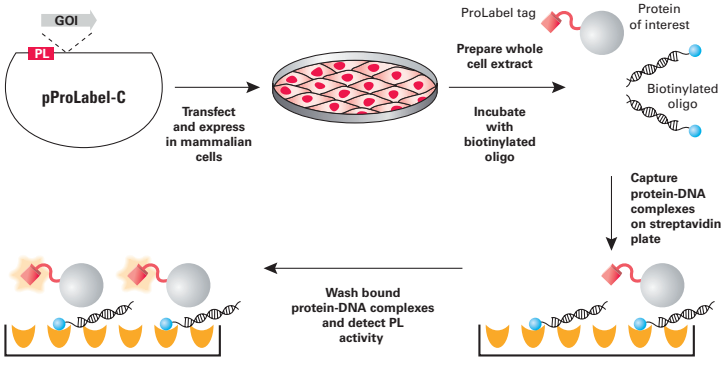



**Figure 2. Schematic diagram of the Protein-DNA Binding Assay.** PL = ProLabel. GOI = gene of interest.

# I. Introduction & Protocol Overview continued

## Specific & Quantitative Detection of DNA-Protein Interactions

To verify that the Protein-DNA Binding Assay detects specific interactions, we compared the relative binding activities of ProLabel-p53 and ProLabel-Lamin fusion proteins after each was separately incubated with 5'-biotinylated, annealed oligonucleotides that contained tandem repeats of the wild-type (WT) p53 *cis*-acting DNA consensus elements (Table I). The relative levels of ProLabel activity captured on the plate for each binding reaction (Figure 3) demonstrated that our *in vitro* assay detects specific binding of p53 to its *cis*-DNA consensus binding element. The WT 3X p53-containing oligonucleotide bound 55-fold more ProLabel-p53 than ProLabel-Lamin, as determined by assaying ProLabel activity in the immobilized protein-DNA complexes. These measurements were also quantitative, since the overall level of ProLabel-p53 binding detected was dependent on the amount of whole cell lysate added to each sample.

The assay screens for specificity in terms of the target sequence as well as the ProLabel fusion protein, since 15-fold more ProLabel-p53 binding activity was detected when utilizing the WT 3X p53 oligo instead of a mutated p53 oligo. Moreover, the binding of ProLabel-p53 to the WT 3X p53 oligo could be competed off in the initial incubation step with a non-biotinylated WT 3X p53 oligo (Table I; Figure 4).

**Table I: Oligonucleotide Sequences Used in the DNA-Protein Binding Assays**

| Sequence | Oligo Type |
|---|---|
| **p53 consensus sequence** | |
| RRRCWWGYYYRRRCWWGYYY | wild type |
| RRRAWWGYYYRRRAWWGYYY | mutant |
| where R = A or G, W = A or T, and Y = C or T | |
| **5'-Biotinylated annealed p53 oligos used in the protein-DNA binding assay** | |
| 3 x (AGGCATGCCTAGCATGCCT) | wild type |
| 3 x (AGGAATGCCTAGAATGCCT) | mutant |
| **Competitor p53 oligo** | |
| 3 x (AGGCATGCCTAGCATGCCT) | no biotin |

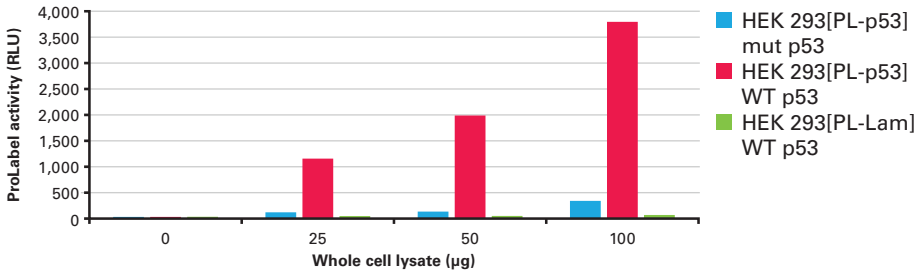



**Figure 3. The Protein-DNA Binding Assay quantitatively detects specific binding of ProLabel-p53 fusion protein to wild-type 3X p53 oligo.** Variable amounts of whole cell lysates (prepared In TALON® Extractor Buffer) containing mammalian-expressed ProLabel-Lamin fusion protein (negative control) or ProLabel-p53 fusion protein (positive control) were incubated with either a 5'-biotinylated, wild-type, 3X p53 annealed oligo or a mutated version. The overall protein levels of the lysate containing the expressed ProLabel-Lamin or ProLabel-p53 fusion proteins were assayed by the BCA method, to normalize for the addition of equivalent amounts of total protein in comparative assays. The indicated amount of each lysate was incubated on ice for 15 min in the presence of Poly dIdC with either the wild-type (WT) 3X p53 biotinylated oligo or the mutated (MUT) 3X p53 biotinylated oligo, allowing protein-DNA complexes to form. The protein-DNA complexes were then transferred and immobilized onto a streptavidin-coated 96-well plate by incubation at room temperature for 1 hr. After washing the wells 4X with Clontech's 1X TransFactor buffer, ProLabel activity was assayed to measure the binding of ProLabel-p53 and ProLabel-Lamin to the oligos.

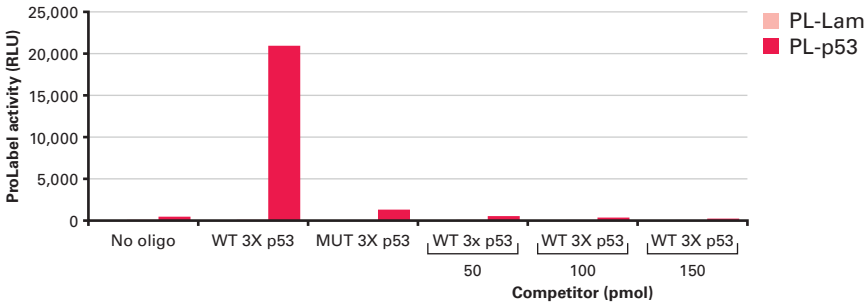



**Figure 4. A competition assay confirms the specificity of ProLabel-p53 binding to wild-type 3X p53 oligo.** The interaction of ProLabel-p53 with the WT 3X p53 oligo is specific and can be competed off by adding a nonbiotinylated competitor oligo to the initial binding reaction.

# II. List of Components

The **Protein-DNA Binding Assay** (Cat. No. 630460) contains sufficient reagents for 96 rxns.

Store the TALON Extractor Buffer, blocking reagent and the streptavidin plate at 4°C. The 10X TransFactor Buffer may be aliquoted into smaller, more convenient volumes and stored at –20°C along with all other reagents.

- **40 µl PL AD FWD Primer (10 µM)**

  5'-GAATTCTGCAGTCGACGCCGCC**GAGTACCCATACGACGTACCAGAT**

  Forward PCR primer for amplification of any cDNA sequence from Clontech's Gal4 AD-based yeast one- or two-hybrid pGADT7-prey vector for In-Fusion 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 Primer (10 µM)**

  5'-TAGATCCGGTGGATCC**AACTTGCGGGGTTTTTCAGTATCTACGATT**

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

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

  4.1 kb cloning vector used to express an N-terminal ProLabel-protein fusion in mammalian cells.

- **20 µl pProLabel-p53 Control Vector (500 ng/µl)**

  5.4 kb control vector that expresses an N-terminal ProLabel-tagged p53 transcription factor.

- **15 ml TransFactor Buffer (10X)**

  Specially formulated buffer used in the Protein-DNA Binding Assay.

- **50 µl Poly dIdC (1 mg/ml)**

- **5 µl Control Annealed WT p53 oligo (20 µM)**

  Annealed oligonucleotide with three tandem repeats of the wild-type (WT) p53 cis-DNA consensus binding elements which the ProLabel-p53 fusion protein recognizes and binds to. This oligo is to be used as part of the positive control when performing the assay.

- **5 µl Control Annealed Mutant p53 oligo (20 µM)**

  Annealed oligonucleotide with three tandem repeats of the mutant p53 cis-DNA binding elements to which the ProLabel-p53 fusion protein has reduced recognition and binding. This oligo should result in reduced p53 binding and yield a reduced signal as compared to the WT p53 oligo.

# II. List of Components continued

- **3 g** **Blocking Reagent**

  Reagent to be prepared by rehydration with 1X TransFactor Buffer and used in blocking the streptavidin plate and the Protein-DNA Binding Assay.

- **5 ml** **TALON® Extractor Buffer**

  Specially formulated buffer used to prepare whole cell extract.

- **1** **Streptavidin plate (96-well)**

  96-well streptavidin-coated plate used to capture biotinylated dsDNA-protein complexes for ProLabel detection.

- pProLabel-C Vector Information (PT3935-5)

- pProLabel-p53 Vector Information (PT3989-5)

- **1** **ProLabel Detection Kit II** (also available separately as Cat. No. 631629)

  Reagents for detection of ProLabel activity from the captured dsDNA-ProLabel fusion protein complex:

  – 4 ml Cell Lysis Buffer
  – 3 ml CL Substrate Diluent
  – 0.16 ml Galacton-Star® Substrate*
  – 0.8 ml Emerald-II™ Solution
  – 4 ml EA Reagent
  – 0.1 ml Positive Control Peptide

  *Centrifuge vial before opening.

# III. Additional Materials Required

- DMEM
- FBS
- Sodium pyruvate
- PBS
- Trypsin/EDTA
- CalPhos™ Mammalian Transfection Kit (Cat. No. 631312; recommended) or other transfection reagents
- Cell scrapers
- Halt™ Protease Inhibitor Cocktail (Pierce Biotechnology, Cat. No. 78410) or an analogous substitute
- PMSF
- Customer-specific biotinylated annealed oligo(s)
- Distilled water
- Pipettor
- Pipette tips
- Multi-channel pipet
- Whatman filter paper (folded grade 113V; Whatman Cat. No. 1213-125)
- Luminometer/plate reader
- 24-well plates
- 96-well plate with clear bottom and white/black sides
- Thermocycler

# IV. Preparing & Testing Binding Oligos & ProLabel Fusion Constructs

***PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING***

***Use this procedure to design oligos for the binding assay, clone your protein of interest, and confirm that it is expressed in mammalian cells.***

Protocol

## A. Protocol: Design and Synthesis of Specific DNA-Binding Oligos

### 1. Oligo Purity Requirements

The synthesized oligonucleotides should, at the minimum, be supplied in a desalted form. However, we highly recommend that they be HPLC- or PAGE-purified, particularly if they are longer than 75 nucleotides.

### 2. Wild-Type Binding Oligo Design Guidelines

- Each DNA-binding oligo should contain a specific transcription factor/protein binding consensus sequence flanked by a short sequence on both the 5' and 3' ends. The flanking region should consist of a short DNA sequence ranging from 3–10 base pairs, which does not contain binding sites for other transcription factors. If you are interested in a certain factor/protein and do not know its consensus binding sequence or are seeking alternative binding sites, the binding sequence information may be obtained from the scientific literature or from transcription factor binding sequence databases:

  Recommended databases include the commercially available TransFac Database from BioBase Biological Databases (Wolfenbüttel, Germany) at **http://www.biobase.de/** and the public databases at **http://www.cbrc.jp/research/db/TFSEARCH.htm** and at **http://www.modor.cgb.ki.se/sgi-bin/jaspar2005/jaspar_db.pl** . In all of these databases, a field labeled MATRIX lists the highly conserved binding sequence for each transcription factor compiled from multiple known binding sequences. The consensus sequence is the portion that is very highly conserved. MATRIX also includes flanking sequences that are not as highly conserved. The public JASPAR database at **http://www.modor.cgb.ki.se/sgi-bin/jaspar2005/jaspar_db.pl** is an open-access database of annotated, high-quality, matrix-based transcription factor binding site profiles for eukaryotes developed by the Center for Genomics and Bioinformatics, Karolinska Instituret, Stockholm, Sweden (Sandelin *et al.*, 2004).

- An oligo that contains 2–3 concatenated/tandem copies of the binding sequence can often produce a stronger binding signal than a single copy. However, increasing the sequence copy number may not necessarily raise the binding efficiency any further (data not shown). We recommend designing and testing two oligonucleotides with varying numbers of concatenated copies of the binding sequence.

### 3. Control Oligo Design Guidelines

- **Mutant Binding Oligos:** Mutant binding oligos may be used as additional controls for the binding assay. To design a mutant binding oligo, replace the most highly conserved nucleotides in the consensus sequence (which are most likely to be the nucleotides that interact directly with the protein/transcription factor) with other nucleotides. To make the mutant oligo a good control, it is best to limit the number of nucleotides that are changed to no more than 4 within a given DNA binding site.
- **Wild-Type Competitor Oligos:** A wild-type competitor oligo has the same sequence as a wild-type binding oligo, but it is not biotinylated.

Protocol
30 min

### 4. Oligo Synthesis and Annealing

- After the DNA-binding consensus sequence is determined, arrange for the synthesis of two complementary oligos. One of the two oligos should contain a biotin label at its 5'-end.
- Combine equimolar ratios of the two complementary oligos, each at an approximate concentration of 100 µM, in a volume of 100–500 µl. This will give a theoretical yield of 50 µM of biotinylated annealed oligo pair.
- Heat the oligo mixture at 95°C for 10 min in an Eppendorf tube in a heating block, and then allow the block containing the mixture to cool down slowly to room temperature.
- After diluting the double-stranded oligo to its desired concentration (see Section VI.B), it is ready for use.

# IV. Preparing & Testing Binding Oligos & ProLabel Fusion Constructs continued

Protocol

## B. Protocol: Cloning of ProLabel Fusion Constructs

- Any gene of interest can be inserted into the multiple cloning site of the pProLabel-C Vector to generate a ProLabel fusion construct. It is important that the cloning design yields an in-frame fusion with the ProLabel tag and does not contain any premature stop codons, otherwise the proper ProLabel fusion protein will not be expressed.
- The In-Fusion primers provided in the kit, PL AD FWD/REV, are specifically designed to facilitate the directional PCR cloning of inserts/cDNA from any of the following Gal4 AD-based yeast one- or two-hybrid library vectors (pGADT7, pGADT7-Rec, pGADT7-Rec2, or pLP-GADT7) into the SalI/BamHI restriction sites of the pProLabel-C vector to generate in-frame ProLabel fusion proteins. Please note that this primer set may share sequence homology with other Gal4-based AD vector constructs in addition to those listed here. Please check the boldfaced, underlined portions of the primer sequences (see Section II) 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 gene sequences; however, different In-Fusion primer designs are necessary for the cloning as well as for generating in-frame fusions. It is also possible to use traditional restriction enzyme cloning.
- We recommend Clontech's In-Fusion 2.0 CF Dry-Down PCR Cloning Kit (Cat. No. 639607 or 639608) for simple, efficient, directional PCR cloning of your insert(s) into the pProLabel-C vector. Whether you use In-Fusion 2.0 or an alternative PCR cloning system, it is essential that the DNA polymerase used for the amplification has superior performance and high fidelity, such as Clontech's Advantage® HD Polymerase Mix (Cat. No. 639241) so as to ensure that the function of the expressed ProLabel fusion protein is not compromised by any introduced mutations.

Protocol
2 hr

## C. Protocol (Optional): Amplification of Insert with Universal In-Fusion Primers

If you are using the Universal In-Fusion Primers supplied in this kit for directional In-Fusion PCR cloning of a cDNA insert from any of the following Gal4 AD-based yeast one- or two-hybrid library vectors (pGADT7, pGADT7-Rec, pGADT7-Rec2, or pLP-GADT7), the following set-up and thermocycler conditions are recommended.

### 1. PCR Set-Up

| | |
|---|---|
| 1 µl | pGADT7-cDNA plasmid template (1ng/ul) or water for negative no template control (NTC) |
| 10 µl | 5X Advantage HD PCR Buffer |
| 4 µl | dNTP mix (2.5 mM each) |
| 1 µl | PL AD FWD Primer (10 µM) |
| 1 µl | PL AD REV Primer (10 µM) |
| 32.5 µl | deionized water |
| 0.5 µl | Advantage HD Polymerase |
| 50 µl | Total Volume |

# IV. Preparing & Testing Binding Oligos & ProLabel Fusion Constructs continued

### 2. Thermocycler Program

We recommend the following thermocycler program for use with the primers provided in the kit:

**Cycling Parameters**

98°C for 5 min

30 cycles

98°C for 15 sec

55°C for 15 sec

72°C for 1 min/kb

72°C for 10 min

4°C for ∞


**NOTE:** As a general rule of thumb, the extension time should be 1 min/kb, but 3 min will work for the majority of the cDNAs in the Gal4-based pGADT7-AD library. If you know that your cDNA is longer than 3 kb, then change the extension time accordingly.

Analyze 5 µl of the PCR product on a 1% agarose/TAE/EtBR gel alongside a DNA standard, such as a 1 kb ladder, to assess the yield and specificity of the product before proceeding to the cloning step.


**Protocol**
**1 hr**

## D. Protocol: Verifying ProLabel Activity from ProLabel Fusion Proteins

Once the ProLabel fusion protein has been generated and a stock DNA solution has been purified, it is important to verify expression of the ProLabel fusion protein in mammalian cells prior to performing your Protein-DNA Binding Assay. The ProLabel activity of your fusion protein can be easily assessed in the lysate from cells transfected the ProLabel fusion construct, using the ProLabel Detection Kit II included with the DNA-Protein Binding Assay.

### 1. Transfection of ProLabel Fusion Constructs

Using a cell line and a transfection reagent of choice, perform the following transfections in a 24-well plate, according to the procedures recommended by the transfection reagent's manufacturer. The following constructs should be independently transfected (GOI = gene of interest):

a. pProLabel-C (negative control)

b. pProLabel-p53 (positive control)

c. pProLabel-GOI


**NOTE:** We typically use calcium phosphate to cotransfect HEK 293 cells (using our CalPhos Mammalian Transfection Kit; see Section III), since this combination consistently yields high transfection efficiencies. If you wish to use a different transfection reagent and/or cell line, make certain that the selected transfection reagent is capable of providing high transfection efficiencies in your particular chosen cell line.

# IV. Preparing & Testing Binding Oligos & ProLabel Fusion Constructs continued

**2. Preparation of ProLabel Assay Reagents**

Forty-eight hr posttransfection, prepare reagents and assay for ProLabel fusion protein expression using the included ProLabel Detection Kit II. First thaw the components from the ProLabel Detection Kit II at room temperature. Once the components are thawed, invert to mix and then place the components on ice.

a. Preparation of ProLabel Detection Buffer

- Combine 1 volume of Cell Lysis Buffer with 3 volumes of EA Reagent. 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 fusion protein expression, you will need 100 µl of ProLabel Detection Buffer per sample to lyse the transfected cells. It is a good idea to prepare 10% extra to account for pipetting error.


**NOTE:** You will use 80 µl of each lysate for the ProLabel assay. (see Section IV.D.3.e).

c. Preparation of Substrate Mix

| Reagents | Volume Per Assay Sample |
|---|---|
| Galacton-Star Substrate | 1.2 µl |
| Emerald II Solution | 6.0 µl |
| CL Substate Diluent | 22.8 µl |
| Total Volume/Sample | 30.0 µl |

**3. Prolabel Detection Procedure**

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 100 µl of the ProLabel Detection 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 a clear bottom and white/black sides.

f. Set up a positive control sample by mixing 50 µl of Positive Control Peptide with 30 µl of ProLabel Detection Buffer and adding the mixture to an empty well.

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

h. Gently pipet up and down twice to mix the contents.

i. Incubate the plate at room temperature from 15 min up to 1 hr.

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

**4. Interpretation of Results**

Different ProLabel fusions will yield different levels of ProLabel activity. However, if your ProLabel fusion protein is efficiently expressed in the transfected cells, then the ProLabel activity detected should be significantly higher than the one observed in the negative control—cells transfected with the empty pProLabel-C Vector—as these lysates should not yield any significant ProLabel activity.

# V. Expression of ProLabel Fusion Protein for the Binding Assay

***PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING***
***Use this procedure to express your ProLabel fusion protein of interest in mammalian cells (Section A) and prepare a whole cell extract (Section B) for use in the Protein-DNA Binding Assay.***

Protocol
1 hr

## A. Transfection of Mammalian Cells

1. For each transfection, you will need one 60 mm plate.
2. One day before the transfection, seed cells onto 60 mm plates at a density recommended by the manufacturer of your transfection reagents. For HEK 293 cells being transfected with Clontech's CalPhos Mammalian Transfection Kit (see Section III), this means that approximately 1 x $10^6$ cells are seeded onto each 60 mm plate.
3. Set up the control and experimental transfections: as follows
   a. Positive Control: pProLabel-p53
   b. Experimental Sample: pProLabel-GOI fusion construct (GOI = gene of interest)
4. Transfect according to the protocols recommended by the reagent's manufacturer.

Protocol
1.5–2 hr

## B. Preparation of Whole Cell Extract (WCE)

**NOTE:** Samples should be kept on ice during the entire extraction procedure to prevent protein degradation and denaturation.

1. Forty-eight hr posttransfection, remove the culture medium and wash the cells in each plate with 2 x 5 ml of cold PBS.
2. Aspirate PBS and place the plates containing the cells on ice.
3. Calculate and prepare the required amount of Cell Extraction Buffer (CBE) as follows and keep it on ice:
   - Prepare 600 µl of CBE per 60 mm plate
   - CBE = TALON Extractor Buffer containing 1X Halt Protease Inhibitor Cocktail (recommended, see Section III) or a similar mixture of protease inhibitors, and 1 mM PMSF.
4. Add 500 µl of CBE to each plate of cells and manually tilt the plate back and forth to coat the surface of the plate with the buffer. Place the remainder of the CBE on ice for use in diluting the sample for the protein determination step (Step 8).
5. Keeping the plate on ice and using a cell scraper, scrape to detach the cells from the culture plate.
6. Collect the loosened cells into a clean 1.5 ml microcentrifuge tube and place the tube on ice for 30 min, vortexing every 10 min for 10 sec to ensure complete lysis.
7. Centrifuge the samples at 20,000 x g for 20 min at 4°C.
8. Transfer the supernatant to a clean 1.5 ml microcentrifuge tube, place the sample on ice and determine the protein concentration using a BCA (bicinchoninic acid) protein assay, or a comparable assay.

   **NOTE:** The whole cell extract (WCE) can be stored at -70°C for up to 1 month in convenient aliquots to prevent multiple freeze-thaw cycles; however, we recommend using the WCE in the binding assay the same day it is prepared to obtain maximal ProLabel signal intensity.

# VI. Protein-DNA Binding Assay

***PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING***
***Use this procedure to perform the Protein-DNA Binding Assay.***

**Protocol**
**10 min**

## A. Preparation of Buffers for Binding Assay

1. Determine the amount of 1X TransFactor (TF) Buffer required:
   (number of assay wells) x 1.5 ml = Total Volume of TF Buffer (needed for both the assay and the washes)

   Dilute the 10X TransFactor Buffer with distilled water to obtain the above volume.

2. Prepare Blocking Buffer as follows:

| | |
|---|---|
| 300 µl/assay | 1X TF Buffer |
| 10 mg/assay | Blocking Reagent* |
| 300 µl/assay | Volume of Blocking Buffer |

   *Mix the Blocking Reagent with the 1X TF Buffer [at a final concentration of 33 mg/ml, or 3.3% (w/v)] until the Blocking Reagent completely dissolves, then filter the Blocking Buffer through Whatman filter paper before use.

3. Keep the remaining 1X TF Buffer on ice to use in the wash steps after the binding assay.

**Protocol**
**2 hr**

## B. Sample Incubation and Immobilization

1. If your sample has been stored at –70°C, thaw the whole cell extract on ice.

   **NOTE:** After the whole cell extract is thawed, we recommend centrifuging the sample at 20,000 x g for 5 min at 4°C to remove residual cell debris. Including this step will decrease the variability of your results.

2. Prepare the sample by mixing the desired amount of whole cell extract and poly dIdC (see Notes below) with 2 pmol biotinylated annealed oligo. In a microcentrifuge tube, adjust the final volume of the mixture to 50 µl with Blocking Buffer.

**NOTES**:

- Optimal extract concentration may vary depending on the protein/transcription factor and cell type. To optimize the assay, perform a dose response curve with your whole cell extract. We find that 100 µg of the control ProLabel-p53 whole cell extract usually provides an adequate signal; however, some extracts may perform better at lower or higher concentrations.
- For a background control, use whole cell extract from cells transfected with the pProLabel fusion construct and omit the biotinylated oligo.
- The biotinylated oligo can be a wild-type or mutant oligo.
- Optimal competitor oligo concentration may vary depending on the transcription factor. For competition assays, add 50 pmol competitor oligo to the sample and reduce the Blocking Buffer volume accordingly to maintain a total assay volume of 50 µl. If this does not generate an adequate decrease, then add more competitor oligo in subsequent competition assays.

# VI. Protein-DNA Binding Assay continued

- The optimal Poly dIdC concentration can vary with different transcription factors. We find that 0.5 µg of Poly dIdC per reaction is a good starting point.

3. Incubate the samples on ice for 15 min.
4. Meanwhile, add 150 µl of the Blocking Buffer (from Section VI.A.2) to each well of the streptavidin plate that will be used in the binding assay and incubate at room temperature for 15 min.
5. Remove the Blocking Buffer from the streptavidin plate.
6. Add the 50 µl sample to the well, and incubate for 60 min at room temperature.
7. Wash the wells 4X with 150 µl of 1X TF Buffer (from Section VI.A.3) per well. Allow 4 min for each wash. After the final wash, remove the 1X TF Buffer from the wells.

**Protocol**
**0.5–1 hr**

## C. ProLabel Detection of Immobilized Protein-DNA Interactions

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

**1. ProLabel Detection Buffer**

- Combine 1 volume of Cell Lysis Buffer with 3 volumes of EA Reagent. Mix well and place on ice until use.
- The volumes can be scaled accordingly depending on how many samples are being assayed. For ProLabel detection of immobilized protein-DNA interactions, you will need 80 µl per binding assay. It is a good idea to prepare 10% extra to account for pipetting error.

**2. Substrate Mix**

| Reagents | Volume Per Assay Sample |
|---|---|
| Galacton-Star Substrate | 1.2 µl |
| Emerald II Solution | 6.0 µl |
| CL Substate Diluent | 22.8 µl |
| Total Volume/Sample | 30.0 µl |

**3. Prolabel Detection Procedure**

a. Add 80 µl of the ProLabel Detection Buffer to each well that contains the captured protein-DNA complex. Then set up a positive control by mixing 50 µl of Positive Control Peptide with 30 µl of ProLabel Detection Buffer and adding the mixture to an empty well.

b. Add 30 µl of Substrate Mix to each well containing the ProLabel Detection Buffer and measure the chemiluminescent signal from each sample using the BD Monolight™ 96-well reader (or equivalent) at 0, 15, 30, 45 and 60 min after addition of substrate.

c. Plot the ProLabel readings as a function of time to qualitatively assess that the signals detected within these time points are within the linear range of ProLabel enzymatic activity.

d. Pick a time point in the linear range that has the highest readings to calculate the signal to noise ratio.

# VII. Troubleshooting Guide

| PROBLEM | POSSIBLE EXPLANATIONS & SOLUTIONS |
|---|---|
| **A. PL AD FWD/REV Primers fail to yield a PCR product** | • PCR component(s) are missing or degraded. |
| | • Template is not one of the GAL4 pGADT7-based library vectors and thus lacks complementarity with the PL AD FWD/REV primers. |
| **B. Low In-Fusion cloning efficiency is observed with PCR product amplified using PL AD FWD/REV primers** | • pProLabel-C Vector is not digested with the correct restriction enzyme; make sure that it is digested with SalI/BamHI when using the PL AD FWD/REV primers for In-Fusion PCR cloning of your insert. |
| | • pProLabel-C Vector is incompletely digested, and the remaining circular or religated single-cut vector can contribute to the background in the cloning. |
| **C. No expression or low expression of the ProLabel fusion protein** | • Lack of expression is often due to the ProLabel fusion protein being out of frame. Check cloning strategies and primer designs to ensure that the fusion protein is in-frame; use a high-fidelity DNA polymerase for the PCR amplification of your insert to avoid PCR-induced mutations that may result in frame-shift or premature stop codon. Sequence to verify. |
| | • Low levels of expression can be the result of low transfection efficiency. |
| | • The steady-state level of your protein of interest may be naturally low; however, this should not affect the Protein-DNA Binding Assay because the ProLabel assay is highly sensitive, and thus capable of detecting protein-DNA interactions despite low expression levels. However, you may increase the incubation time after transfection for increased ProLabel fusion protein expression. |
| **D. Lack of signal or weak signal in all wells** | • Improper design of binding oligo. Refer to Section IV.A for oligo design instructions. |
| | • Poly dIdC concentration used in the binding assay is too high. Omit Poly dIdC or use a lower concentration in the experiment. |
| | • Insufficient amount of cellular extract in the assay due to low steady state level of the ProLabel fusion protein. Increase the amount of whole cell extract used. |
| | • No activity in the cellular extract. This may be due to improper or inefficient induction of the cells, or improper isolation or storage of the cellular extract. Check the literature for the appropriate cell induction reagent and kinetics. |
| | • Improper preparation of the assay reagents from the stocks in the ProLabel Detection Kit II. |
| | • Insufficient incubation time after the ProLabel detection reagents are added. |
| | • Insufficient number of repeat sequences of the consensus *cis*-DNA binding site. |
| **E. High signal in mutant wells** | • Improper mutant oligo design—the mutant oligo lacks the necessary significant changes, particularly at the conserved nucleotides. Refer to Section IV.A for oligo design instructions. |
| **F. No competition or low competition** | • Improper design of oligo. Refer to Section IV.A for oligo design instructions. Check to make sure there is no biotin label on the competitor oligo, and that the only difference between the competitor and the binding oligo is the lack of a biotin label on the former. |
| | • Insufficient amount of competitor oligo added. |

# VIII. References

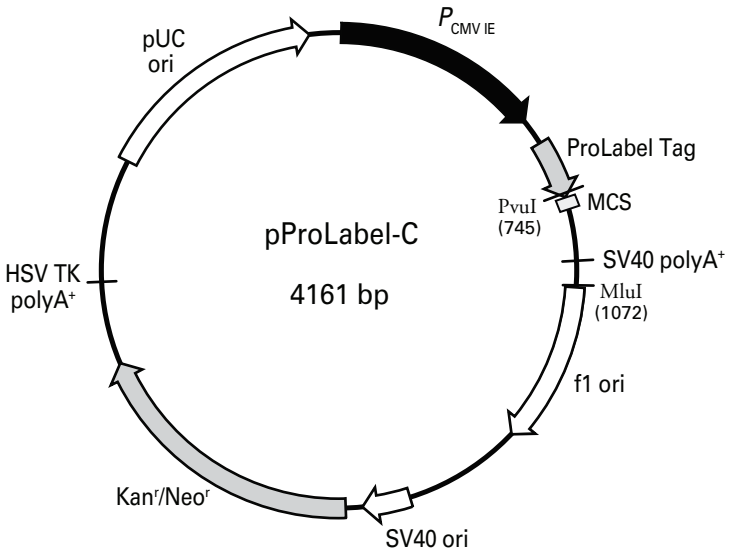
Chemiluminescent Quantification of Protein Expression (July 2007) *Clontechniques* **XXII**(3):18–19.

Tootle, T.L. and Rebay, I. (2005). Post-translational modifications influence transcription factor activity: A view from the ETS superfamily. *BioEssays* **27**(3): 285--298.

Protein-DNA Binding Assay (October 2007) *Clontechniques* **XXII**(4):21–23.

Sandelin, A., Alkema, W. Engstrom, P., Wasserman, W.W., and Lenhard, B. (2004) JASPAR: an open-access database for eukaryotic transcription factor profiles. *Nucleic Acids Res.* **32**: D91-D94.

# Appendix A: Plasmid Information




781 CAA GCT TCG AAT TCT GCA GTC GAC GGT ACC GCG GGC CCG GGA TCC ACC GG

PstI KpnI SmaI

HindIII EcoRI SalI ApaI BamHI

**Figure 5. pProLabel-C Vector Map and Multiple Cloning Site.** pProLabel-C is a mammalian expression vector designed to express a protein of interest, fused at its N-terminus to the the C-terminus of a 6 kDa ProLabel tag. The resulting fusion protein can be quantified using the ProLabel Detection Kit II included with the Protein-DNA Binding Assay (Cat. No. 630460) to perform enzyme fragment complementation assays (1, 2). In these assays, two inactive enzyme fragments (the ProLabel tag, and a larger Enzyme Acceptor) are combined to form a complete, active enzyme that cleaves the Galacton-Star® chemiluminescent substrate. The resulting signal can be detected and quantified with any standard luminometer. The pProLabel-C vector contains a CMV promoter that drives strong, constitutive expression of the fusion protein, and an SV40 polyadenylation signal that directs processing of the 3′ end of the mRNA transcript. The vector also contains a kanamycin/neomycin resistance cassette (Kan$^r$/Neo$^r$) that allows G418 selection of stably transfected eukaryotic cells; a bacterial promoter upstream of this cassette allows kanamycin selection of transformed bacterial cells. In addition, pProLabel-C contains an SV40 origin of replication for propagation in mammalian cells that express SV40 T-antigen, a pUC origin for propagation in *E. coli* and an f1 origin for the production of single-stranded DNA.

The pProLabel-C vector is used to create a fusion of your protein of interest and the ProLabel tag for use in the Protein-DNA Binding Assay in order to detect specific binding of this protein to a biotinylated dsDNA oligonucleotide containing a putative consensus binding sequence. In order to do so, your gene of interest must be in the same reading frame as the ProLabel tag sequence, with no intervening stop codons. ProLabel vector constructs can be transfected into mammalian cells using standard transfection methods. Specific protein-DNA binding interactions can be measured quantitatively from mammalian cell lysates using the instructions in this user manual.

# Appendix A: Plasmid Information continued

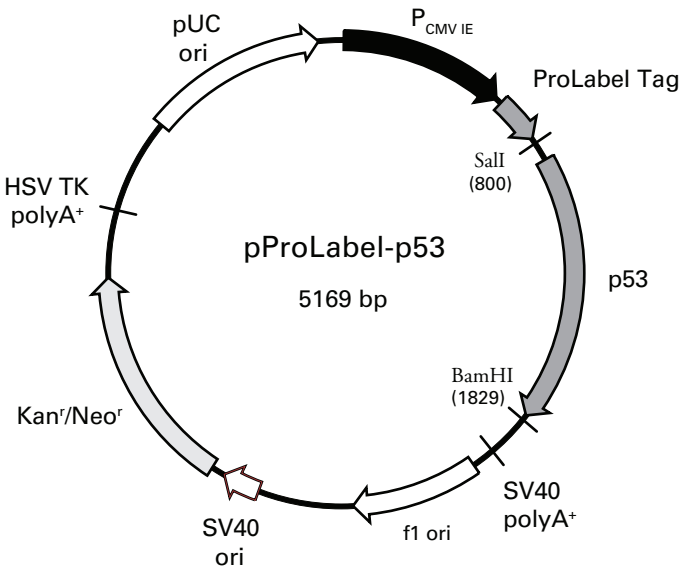



**Figure 6. pProLabel-p53 Vector Map.** pProLabel-p53 is a mammalian expression vector encoding a ProLabel-p53 fusion protein. It expresses the ProLabel tag (~6 kDa) fused to the N-terminus of a truncated version of the murine p53 tumor suppressor protein (containing amino acids 72–391). The resulting fusion protein can be quantified by using the ProLabel™ Detection Kit II included with the Protein-DNA Binding Assay (Cat. No. 630460) to perform enzyme fragment complementation assays (1, 2). In these assays, two inactive enzyme fragments (the ProLabel tag, and a larger Enzyme Acceptor) are combined to form a complete, active enzyme that cleaves the Galacton-Star® chemiluminescent substrate. The resulting signal can be detected and quantified with any standard luminometer. The pProLabel-p53 vector contains a CMV promoter that drives strong, constitutive expression of the fusion protein, and an SV40 polyadenylation signal that directs processing of the 3'end of the mRNA transcript. The vector also contains a kanamycin/neomycin resistance cassette (Kan$^r$/Neo$^r$) that allows G418 selection of stably transfected eukaryotic cells; a bacterial promoter upstream of this cassette allows kanamycin selection of transformed bacterial cells. In addition, pProLabel-p53 contains an SV40 origin of replication for propagation in mammalian cells that express SV40 T-antigen, a pUC origin for propagation in *E. coli* and an f1 origin for the production of single-stranded DNA.

pProLabel-p53 is used as a control construct in the Protein-DNA Binding Assay to show, via ProLabel detection, that p53 binds more specifically to the annealed biotinylated wild-type p53 cis-DNA consensus element than to the annealed biotinylated mutant p53 cis-DNA consensus element. ProLabel fusion protein expression levels can be measured quantitatively from mammalian cell lysates using the method described in this user manual.

# Notes

**Notice to Purchaser**

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**For CMV Sequence**
The CMV promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 assigned to the University of Iowa Research Foundation.

**For ProLabel™ Detection Products**
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