# *In Vitro and In Vivo Methods to Study Protein:Protein Interactions*

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November, 2011

Promega

# *Presentation Overview*

- Review of Methods to Study Protein:Protein Interactions
- In Vitro (Cell-Free) Protein Expression and Application to Interaction Studies
- HaloTag® Fusion Protein Technology and Protein Interaction Experiments
- Applying These Technologies to the Study of Protein-Protein Interactions
  - Case Studies Illustrating Both Discovery and Verification Applications
- Mammalian Two Hybrid Assays
  - Adapting the Yeast Two Hybrid Assay to Mammalian Cells
  - Case Study: Cdk3 Interaction with ATF1
- Other Applications of In Vitro (Cell-Free) Expression and HaloTag® Technology
- Summary

# *Protein-Protein Interactions are Critical to All Cellular Processes*

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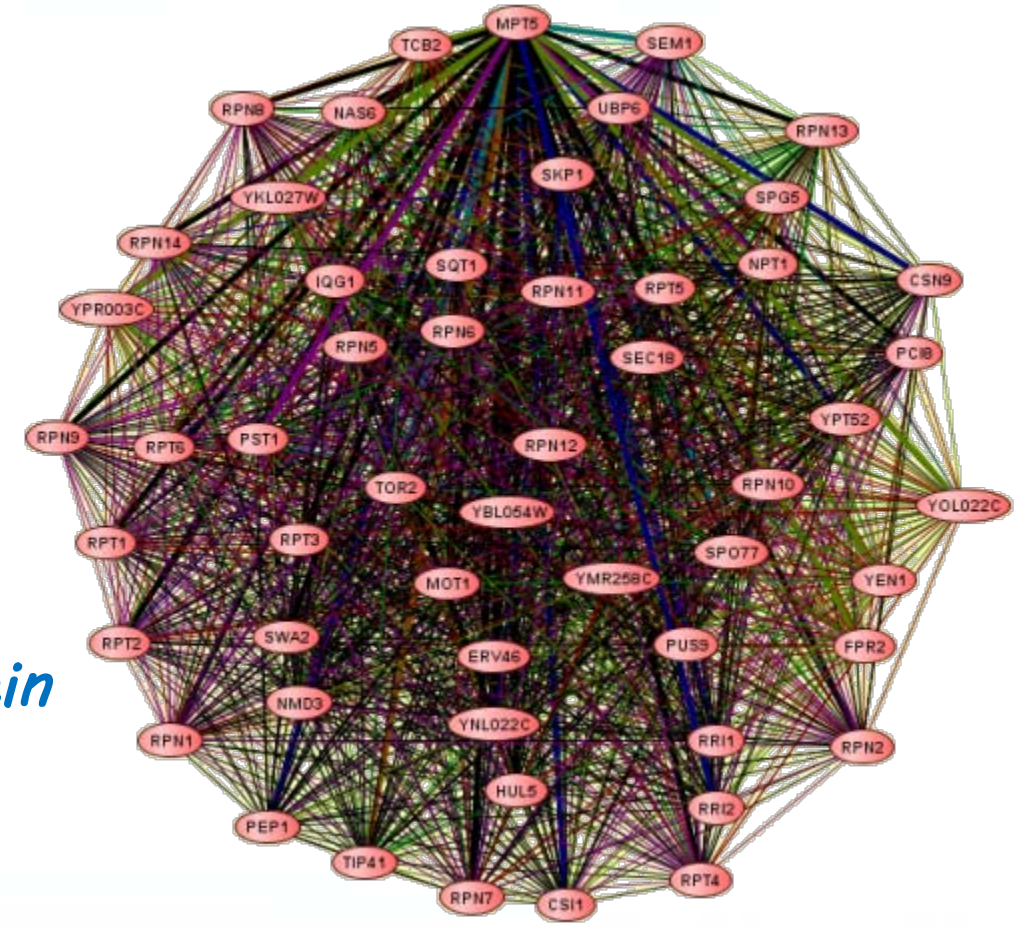
- Replication
- Transcription
- Translation
- Signal transduction & more




***How are protein:protein interactions studied?***

# *Common Methods Used to Study Protein:Protein Interactions*

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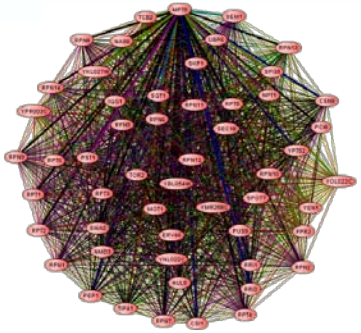
- Co-immunoprecipitation
- Protein Affinity Purification
- Far Western Blotting
- Two Hybrid Assays (yeast, mammalian)
- In Vivo Förster Resonance Energy Transfer (FRET)
- and more…



# *Co-Immunoprecipitation (Co-IP)*
## *A Classic Method to Study Interactions*

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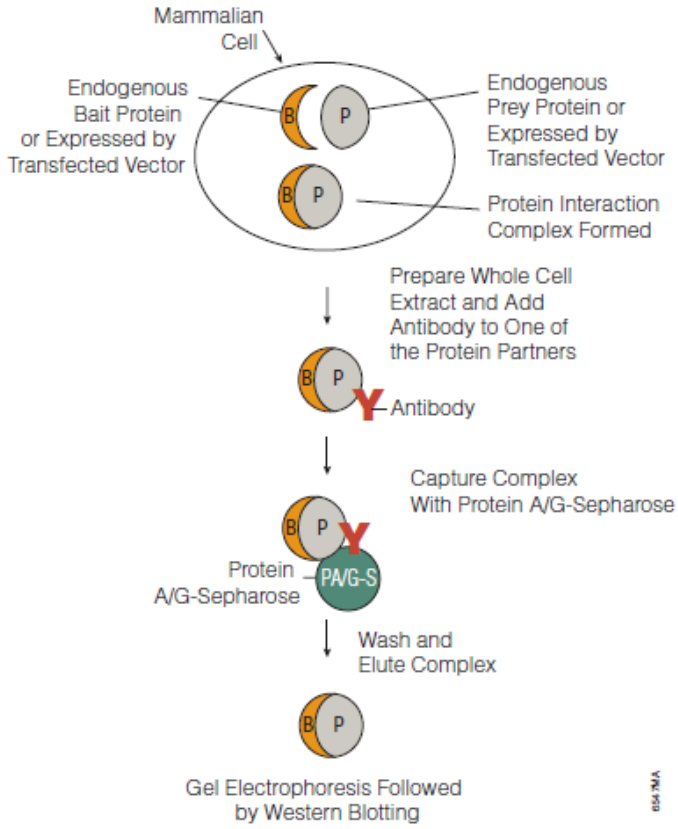
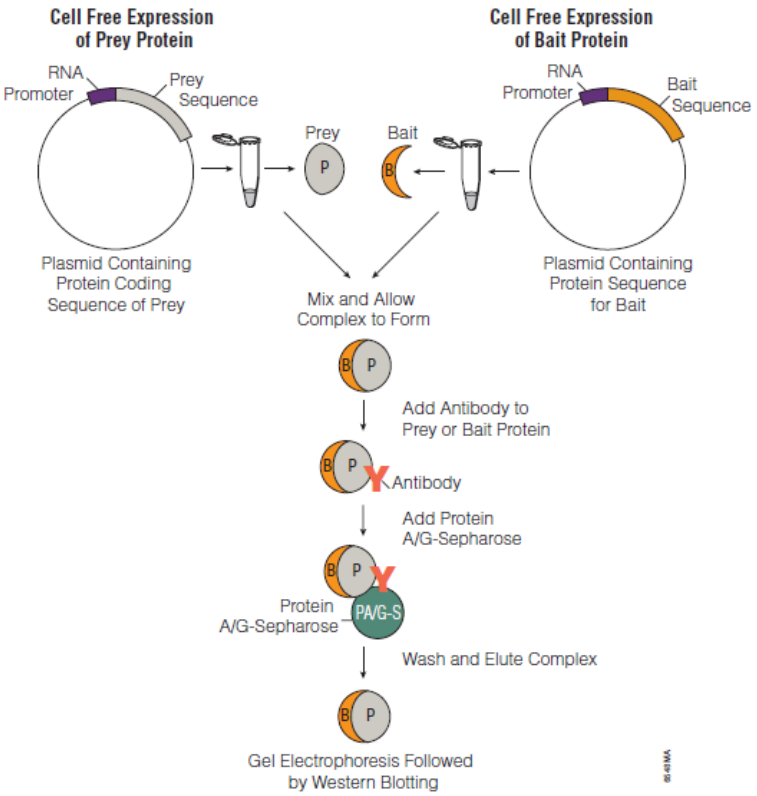
### In Vivo




### In Vitro



# ***Protein Affinity Purification Methods***
## *Identification of Novel Interacting Partners*

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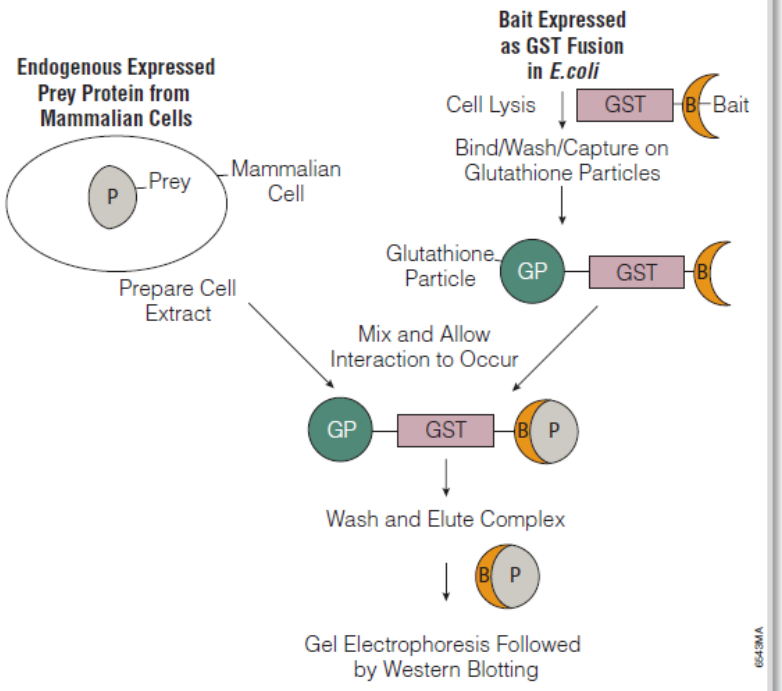
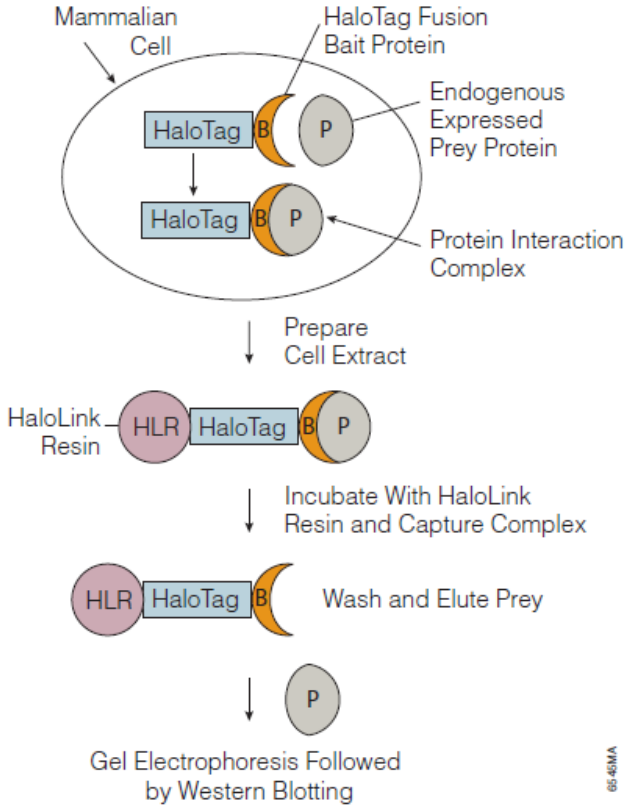
### Purified Bait + Cell Extracts




### In Vivo with Tagged Bait



# ***Protein Affinity Purification Methods***
## *A Quick Method for Verifying an Interaction*

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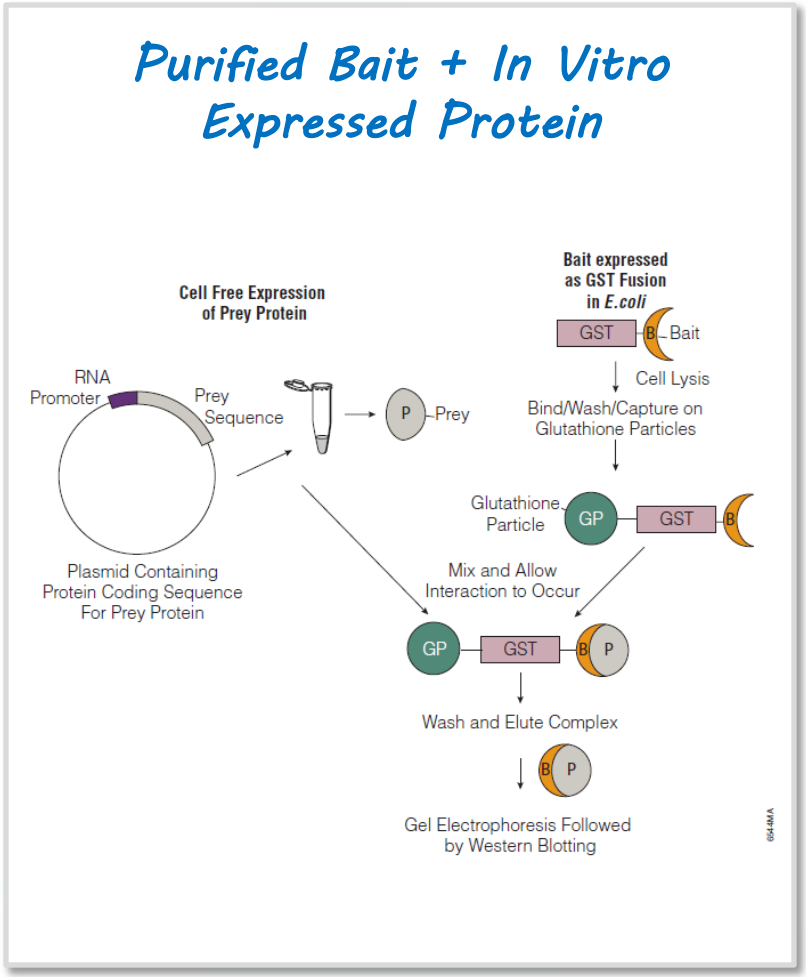
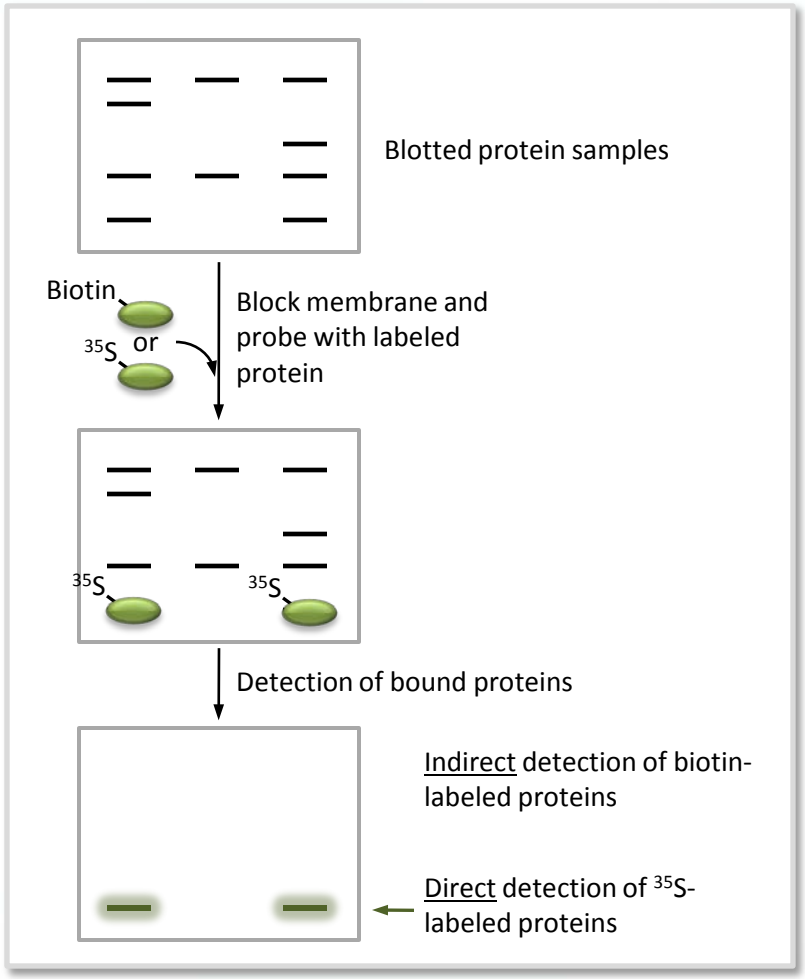


# ***Far Western Blots***
## *A Rapid Method for Testing Interactions*



# ***Two Hybrid and FRET Assays***
*Newer Protein:Protein Interaction Assays*

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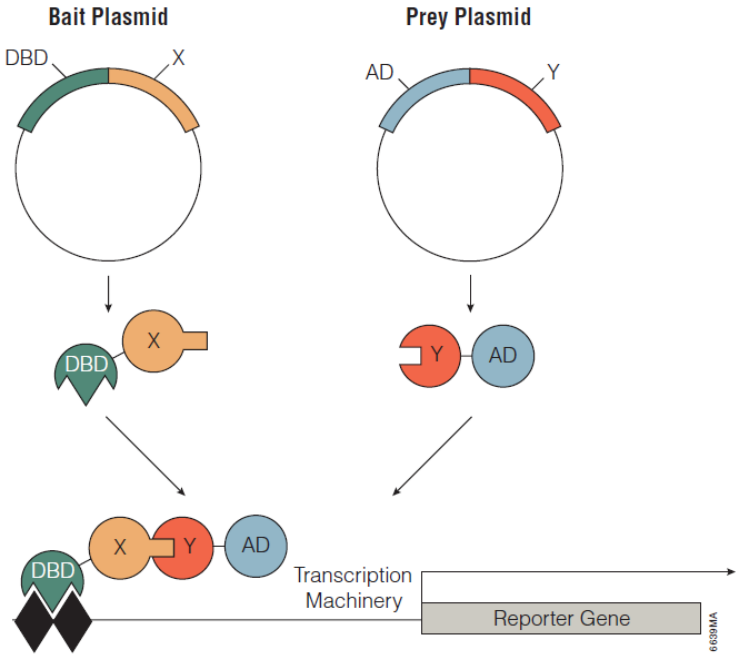
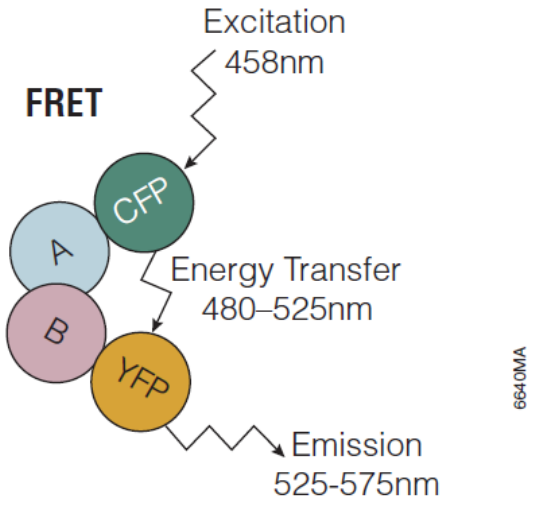
## Two Hybrid Assays




## Förster Resonance Energy Transfer (FRET)



# *In Vitro (Cell-Free) Expression – the Rapid, Easy-to-Use Solution*

In Vitro Production of Target Proteins Using Cellular Extracts for Protein:Protein Interaction Experiments and More

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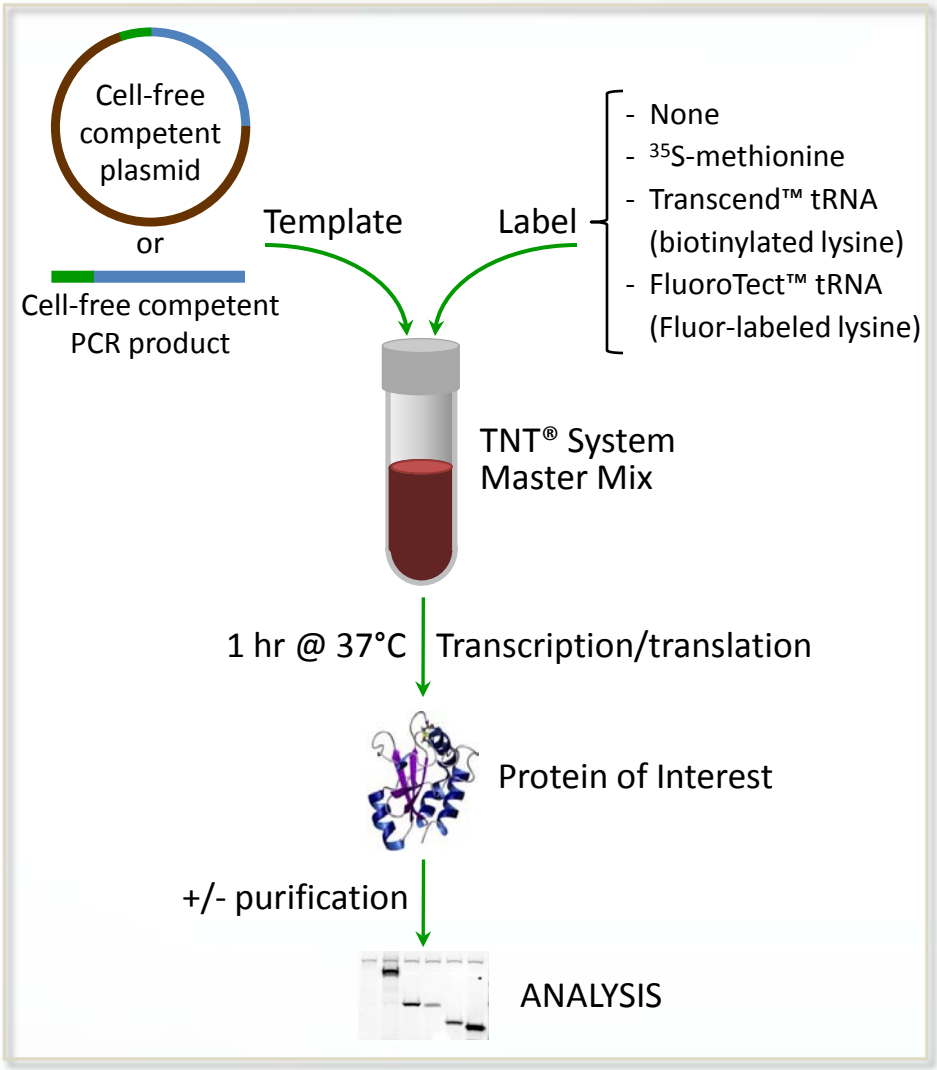
# *Easiest/Fastest Method to Go from DNA to Protein*



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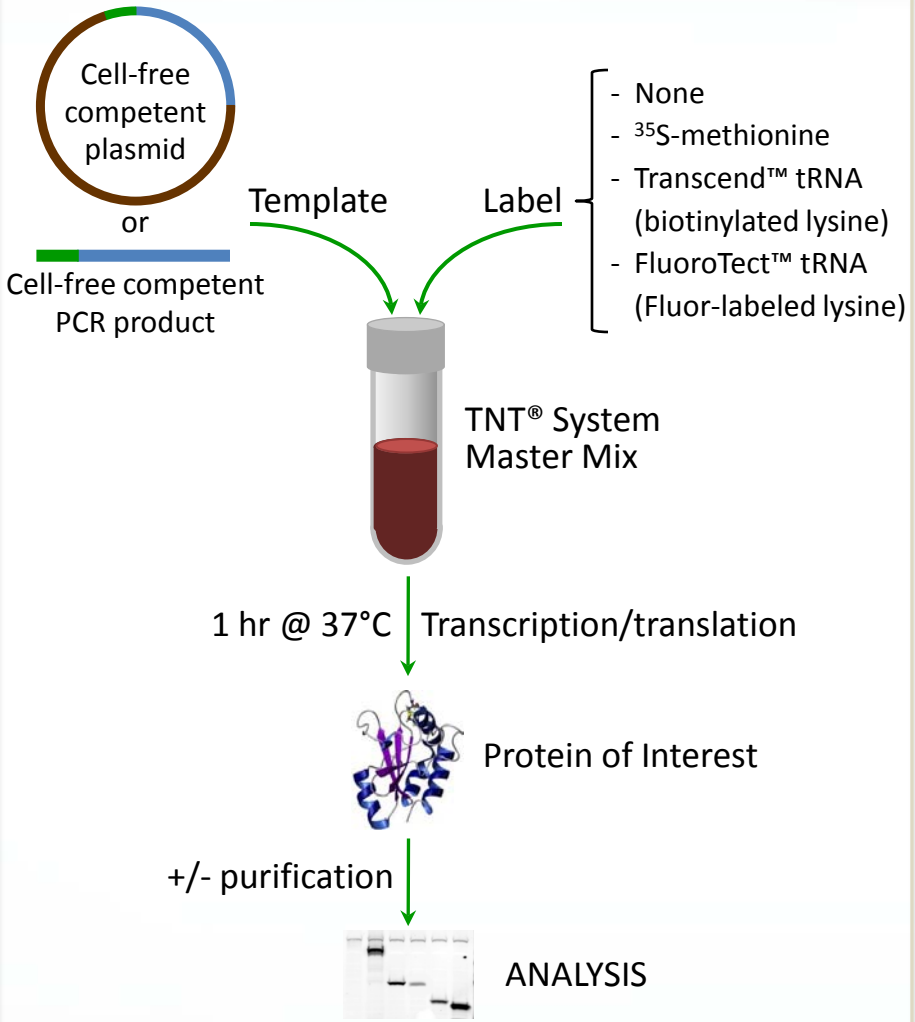
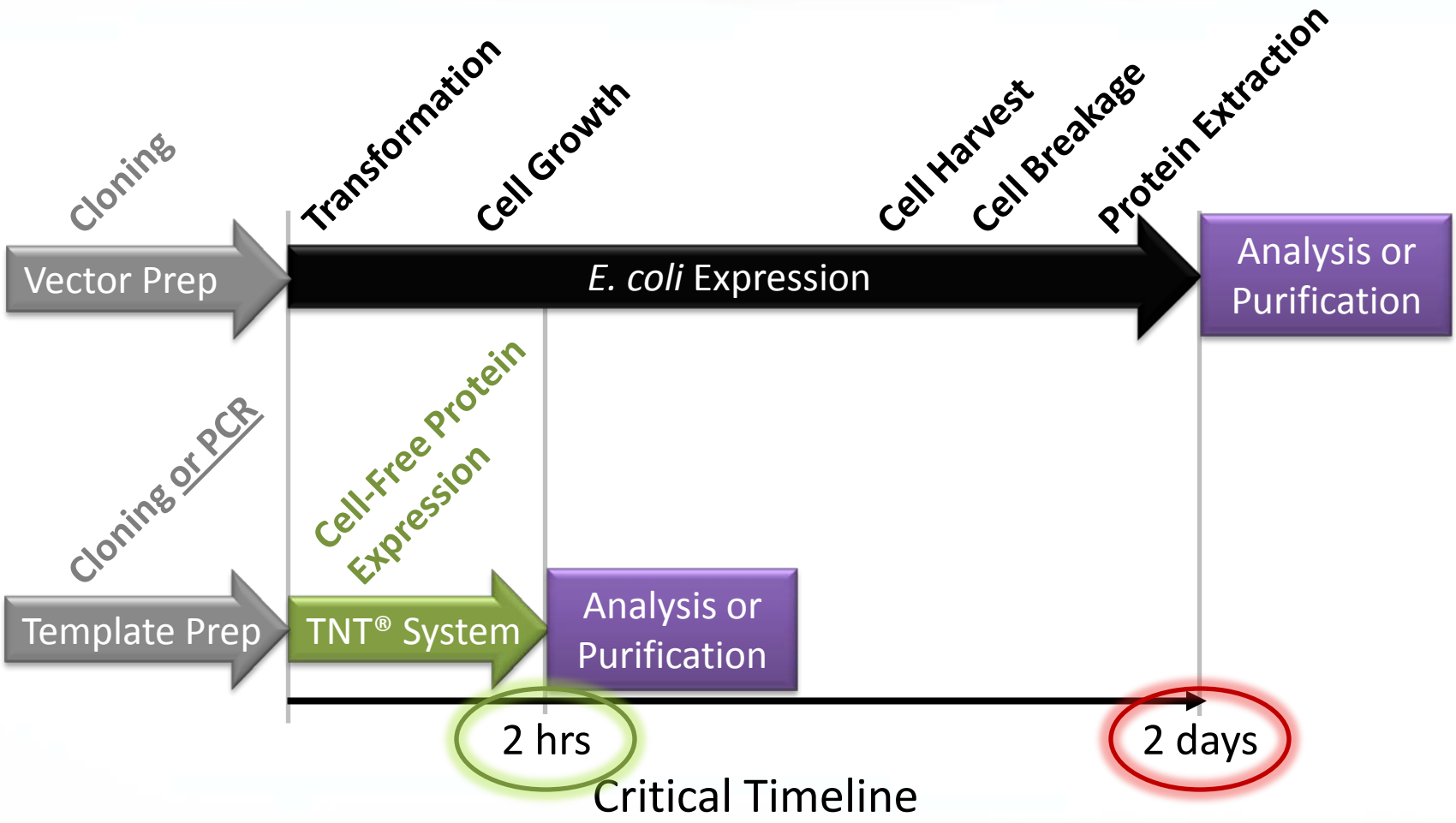



- **Saves valuable time**
  - Produce protein in 1-2 hours vs. days to weeks in *E. coli* or mammalian cells
  - Use PCR product templates and bypass cloning ORF into expression vector
- **Produces sufficient protein for many applications including:**
  - Protein-protein interactions
  - Co-immunoprecipitations
  - Gel-shift assays
  - Enzymatic assays
- **Enables analysis of difficult proteins**
  - Normally toxic to cells
  - Insoluble in *E. coli*
- **Simplified detection**
  - Directly label protein during synthesis
    - Fluorophore, $^{35}$S, biotin

# *Faster Protein Production than E. coli Systems*



# *Choices to Match Your Research Needs*

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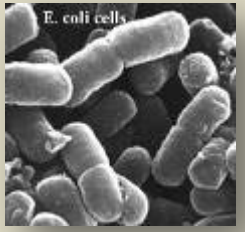
**>75 citations in 2011 Jan-July**

## Prokaryotic

### *Bacteria*




*E. coli*

S30 T7 High-Yield Protein Expression System

Highest Yield

## Eukaryotic

### Plant

Wheat germ

TNT® SP6 High-Yield Wheat Germ System

Maximal soluble protein

### Insect

*Spodoptera frugiperda*

TNT® T7 Insect Cell Extract System

Most active protein

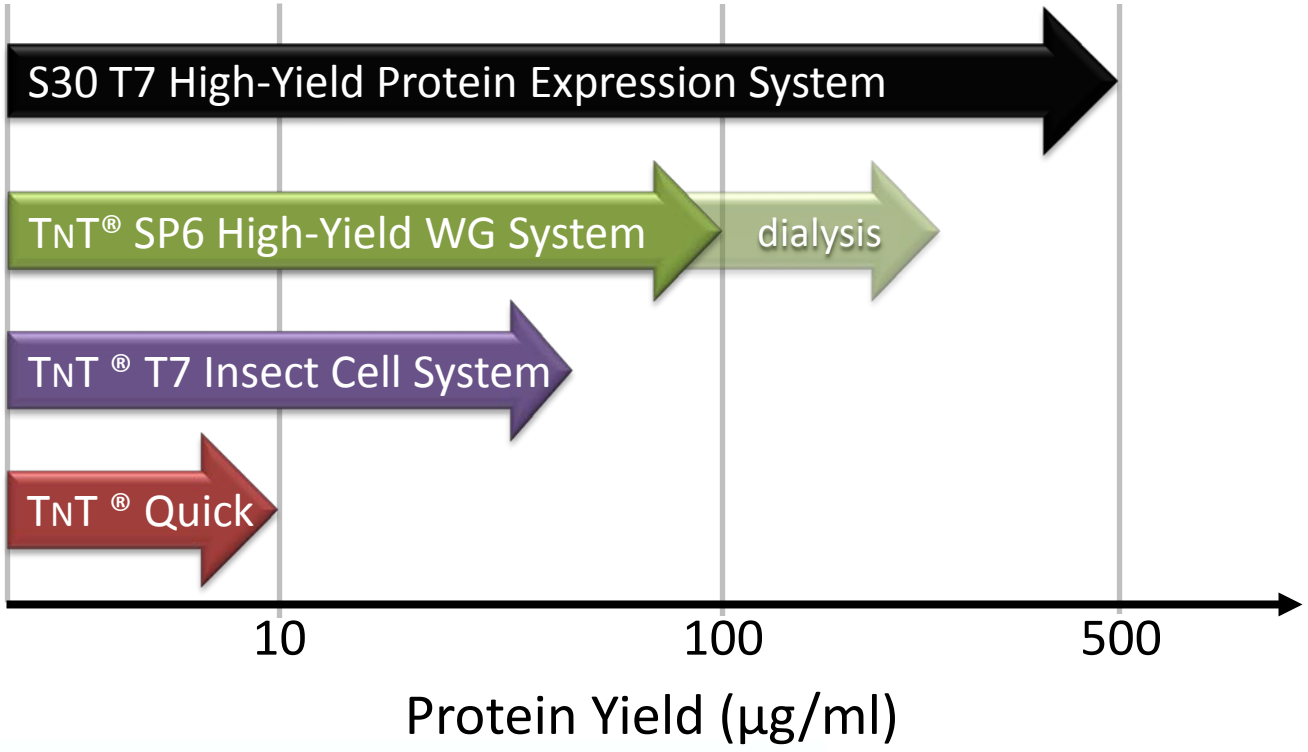
### Mammalian

Rabbit reticulocyte

TNT® Quick Coupled T7 and SP6 Systems

Native mammalian system

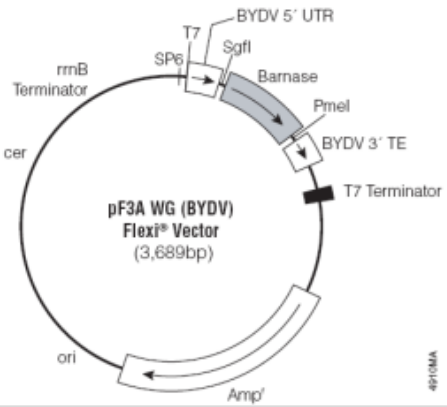
# *Maximal Yields from Each System*



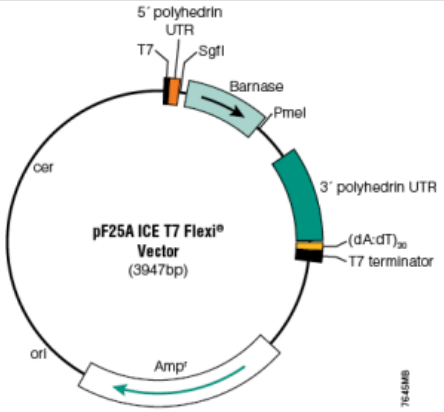
# *Characteristics of In Vitro Expression Systems*

| System | Time | Yield | Requirements |
|---|---|---|---|
| **TNT® T7 or SP6 Quick Coupled System** | 1 hour | ≤0.5µg/50µl | **Any vector** containing a T7 or SP6 promoter upstream of coding sequence. |
| **S30 High Yield (Bacterial)** | 1 hour | ≤25µg/50µl | T7 promoter-driven bacterial expression vector. Can also use very active bacterial promoters (T5) |
| **TNT® SP6 High-Yield Wheat Germ System** | 2 hours | ≤5-12.5µg/50µl | Highest yield with specialized vector containing plant viral sequences (≤5.0µg/rxn). Greatest yield using dialysis method (≤12.5µg/rxn). |
| **TNT® T7 Insect Cell System** | 4 hours | ≤4.0µg/50µl | Require use of a baculovirus expression vector with T7 promoter upstream. |

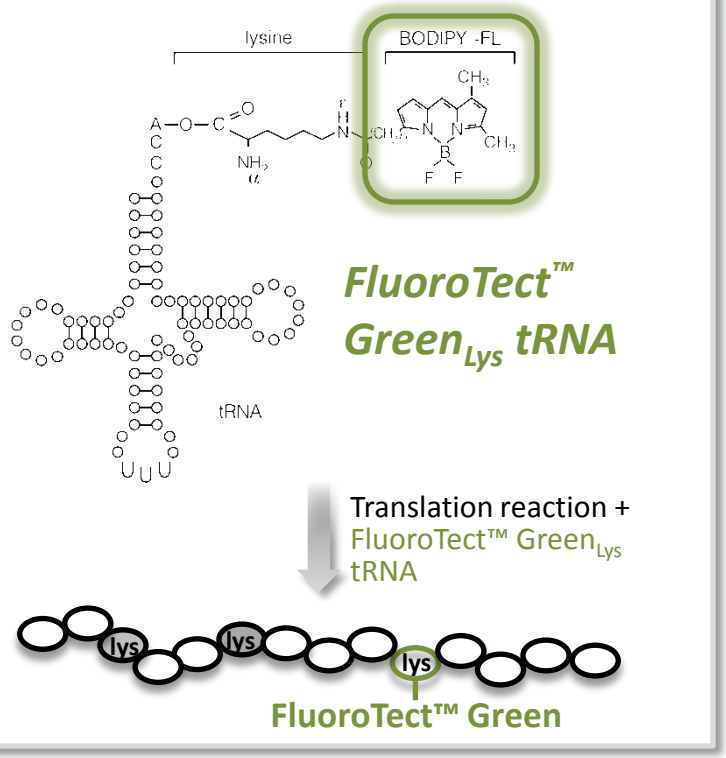
*Optimized Wheat Germ Vector*



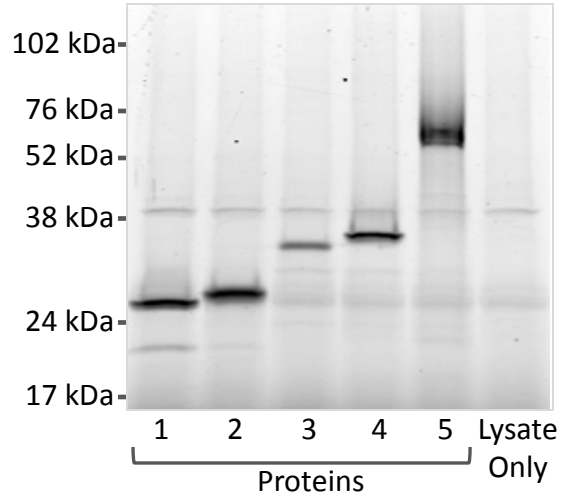

*Insect Cell Vector Example*



# *Fluorescent Detection of In Vitro Expressed Proteins*
## *Non-Radioactive Co-translational Labeling*

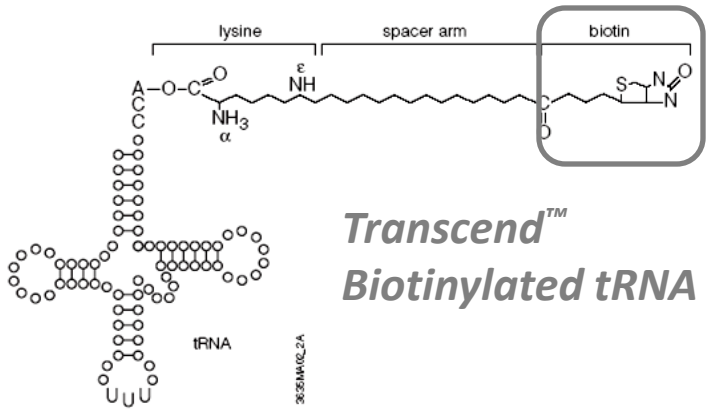



### *TNT® T7 Quick Coupled System*

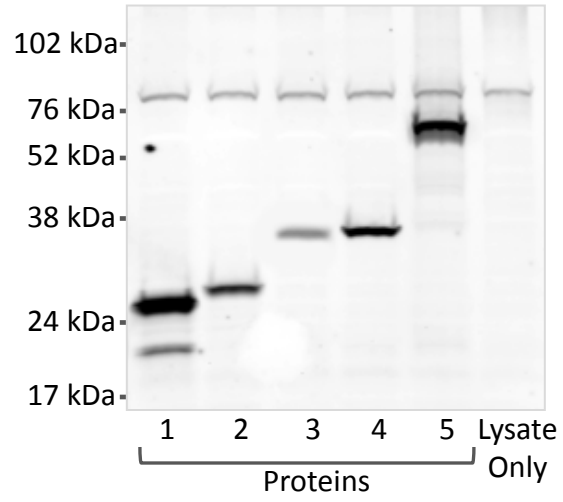



- ✓Produce active/detectable proteins without radioactivity
- ✓Direct detection of fluorescently labeled proteins in gels
- ✓ Use in many applications including pulldowns, co-immunoprecipitations, mobility shift assays…

# *Indirect Detection of In Vitro Expressed Proteins*
## *Transcend™ Biotin Co-translational Labeling*




### *TNT® SP6 Wheat Germ High Yield System*




- ✓Produce active/detectable proteins without radioactivity
- ✓Indirect detection using streptavidin conjugates (HRP, AlkPhos) of biotin labeled proteins
- ✓ Use in many applications including pull downs, co-immunoprecipitations, mobility shift assays…

# *What if There is a Lysine in the Active Site*
## *No Problem – Only 25-33% of Lysines are Labeled*

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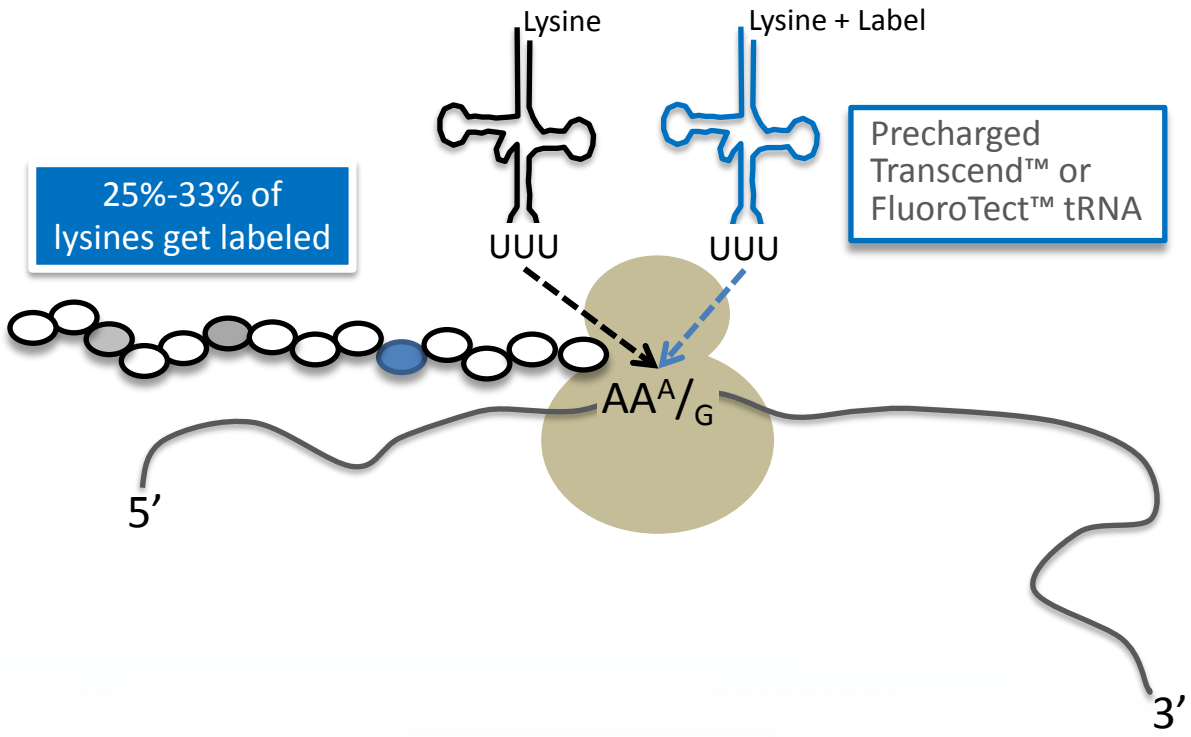
*FluoroTect™ or Transcend™ tRNA compete with natural lysyl tRNA for incorporation into growing peptide chain*



# *HaloTag® Fusion Protein*

A Unique, Multifunctional Fusion Tag Well-Suited to Protein:Protein Interaction Studies

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# *What is HaloTag® Technology?*
## *A Unique, Multifunctional Protein Fusion Tag*

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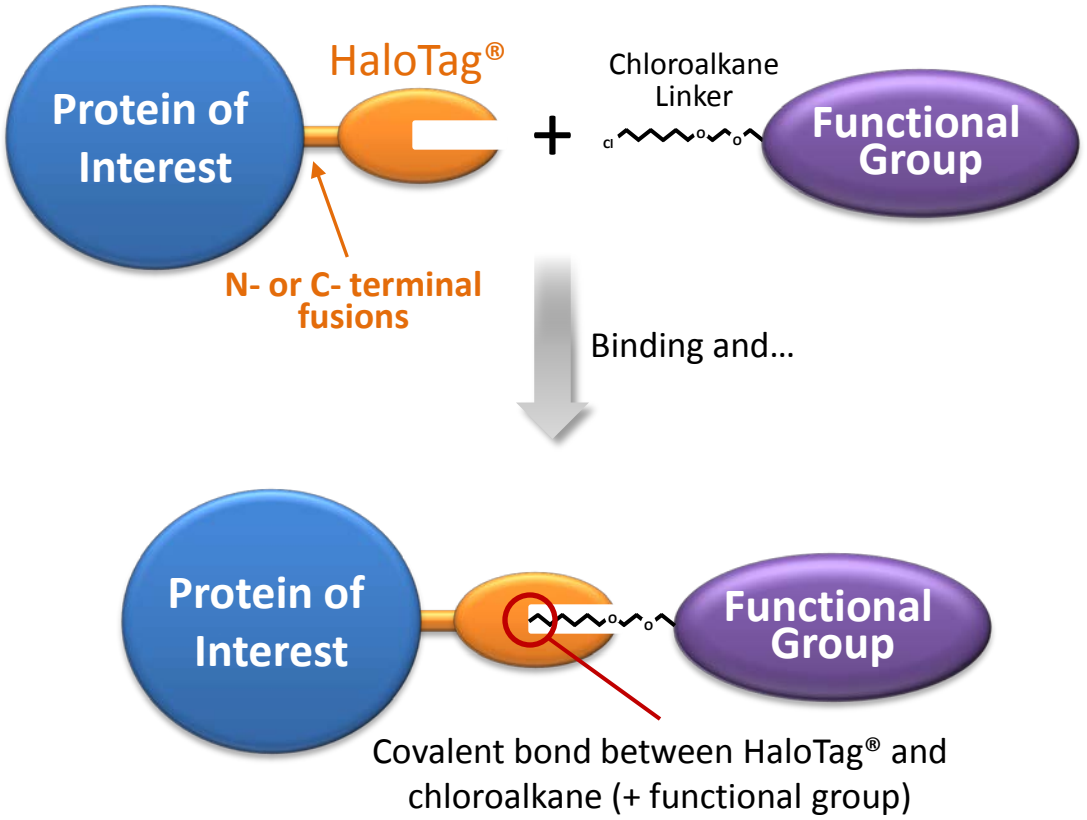
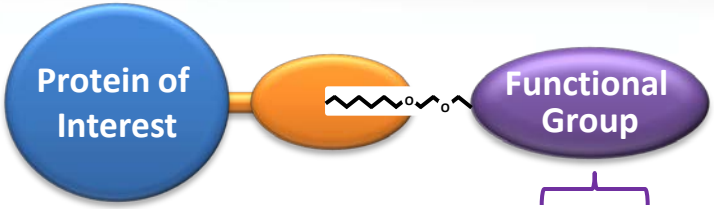
HaloTag®:

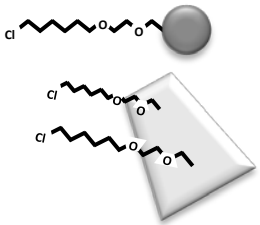
- Engineered 34.1kDa halophilic bacterial hydrolase
- Binds to chloralkane substrate and locks with covalent attachment
- Faster kinetics than the biotin:streptavidin interaction
- No homolog in mammalian cells = no background

Read more about the development of this powerful fusion tag: Ohana, R.F., *et al.* (2009) *Prot. Exp. Purif.* **68**, 110-120.



# *Many Functional Groups are Available to Match Your Research Application(s)*

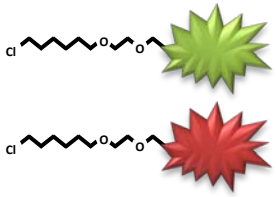


## HaloTag® Surfaces/Resins

*Capture and Display*

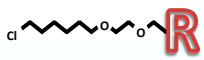
- ✓Protein arrays
- ✓Purification
- ✓Interaction analysis



## HaloTag® Fluorescent Ligands

*Labeling and Detection*

- ✓Cellular imaging
- ✓Gel analysis
- ✓Quantitation




## HaloTag® Reactive Ligands

*Custom Modifications*

- ✓Attach to particles, surfaces
- ✓Attach special ligands

# *One HaloTag® Fusion Protein = Global Protein Characterization Capabilities*

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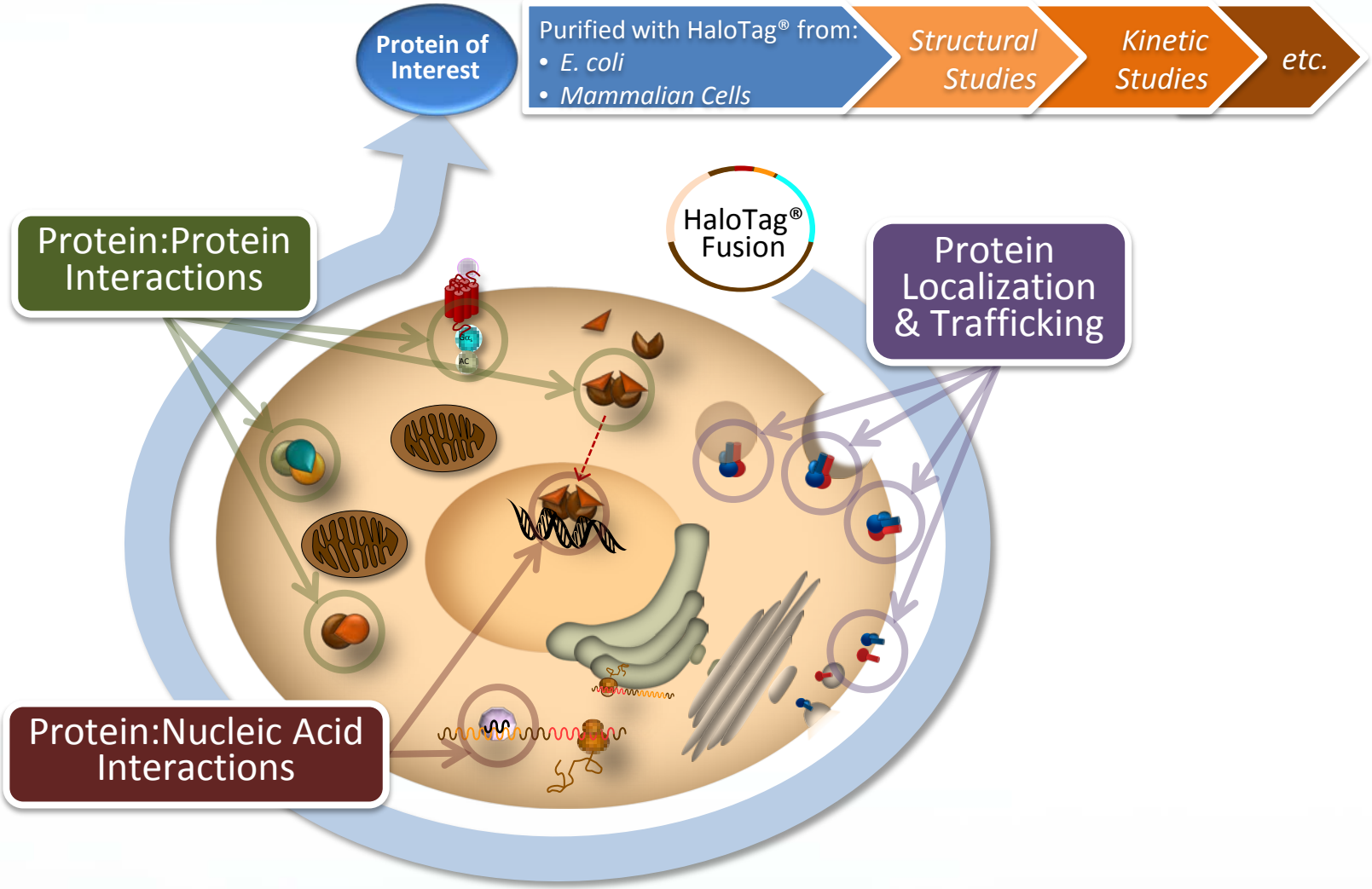
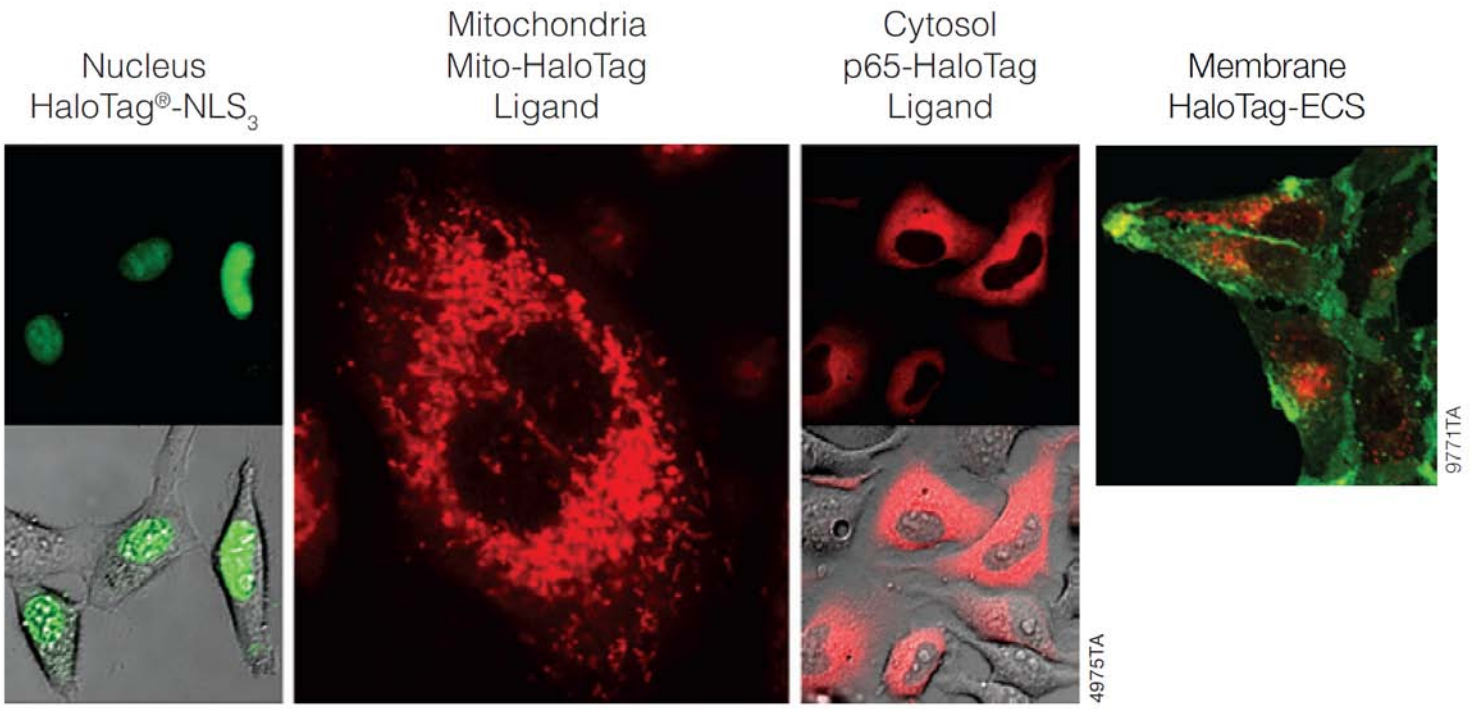
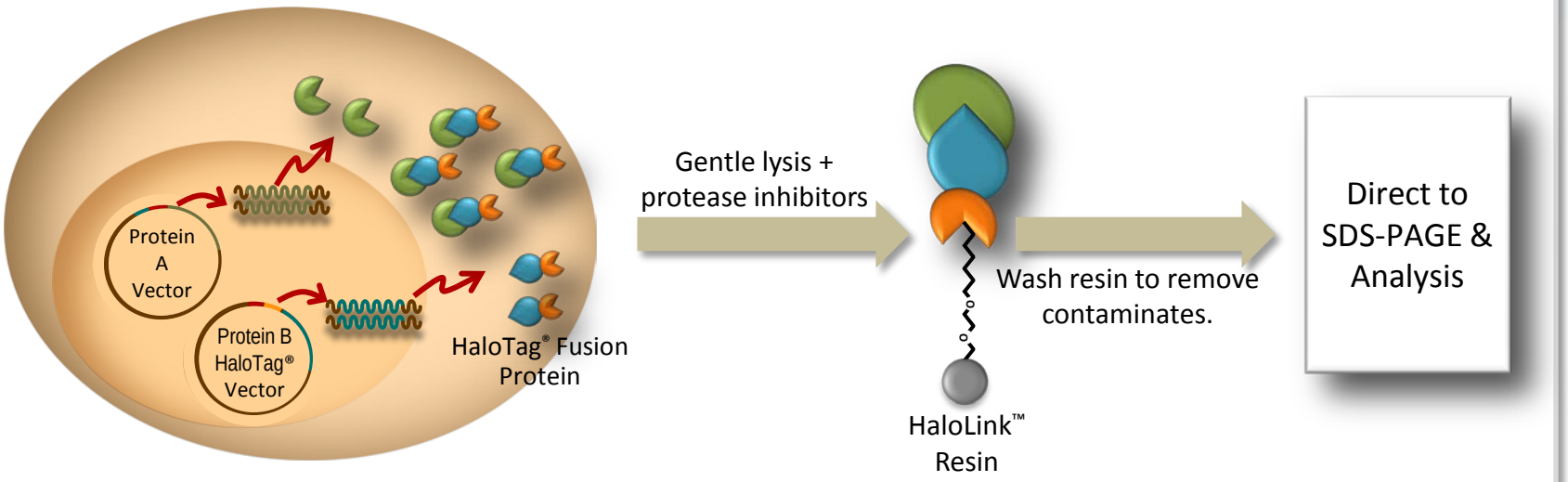


# ***HaloTag® Fusions Go & Are Detectable Anywhere***

*Examples Using Various Fluorophore Ligands*

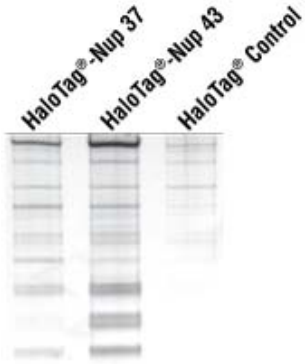


# *Simple, Effective Mammalian Pulldown Assays*
## *Affinity Purification of the Nup 107-160 Complex*




## Capture of Proteins Associated with HaloTag®-Nup 37 and -Nup 43 Fusions

Gel




### Proteins Identified by LC/MS/MS Analysis

| HaloTag®-Nup 37 | HaloTag®-Nup 43 | HaloTag® Control |
|---|---|---|
| Nup 160 | Nup 160 | No Nup subunits detected |
| Nup 133 | Nup 133 | |
| Nup 107 | Nup 107 | |
| Nup 98/96 | Nup 98/96 | |
| Nup 85/75 | Nup 85/75 | |
| Nup 43 | Nup 43 | |
| Nup 37 | Nup 37 | |
| Nup TPR | Nup TPR | |
| NUDC | | |


Méndez, J., et al. (2010) [Internet] [cited: 2011;July 20]. Available from: http://www.promega.com/resources/articles/pubhub/efficient-isolation-identification-and-labeling-of-intracellular-mammalian-protein-complexes/

# *Easy to Build and Customize Protein Arrays with HaloLink™ Array Slides*

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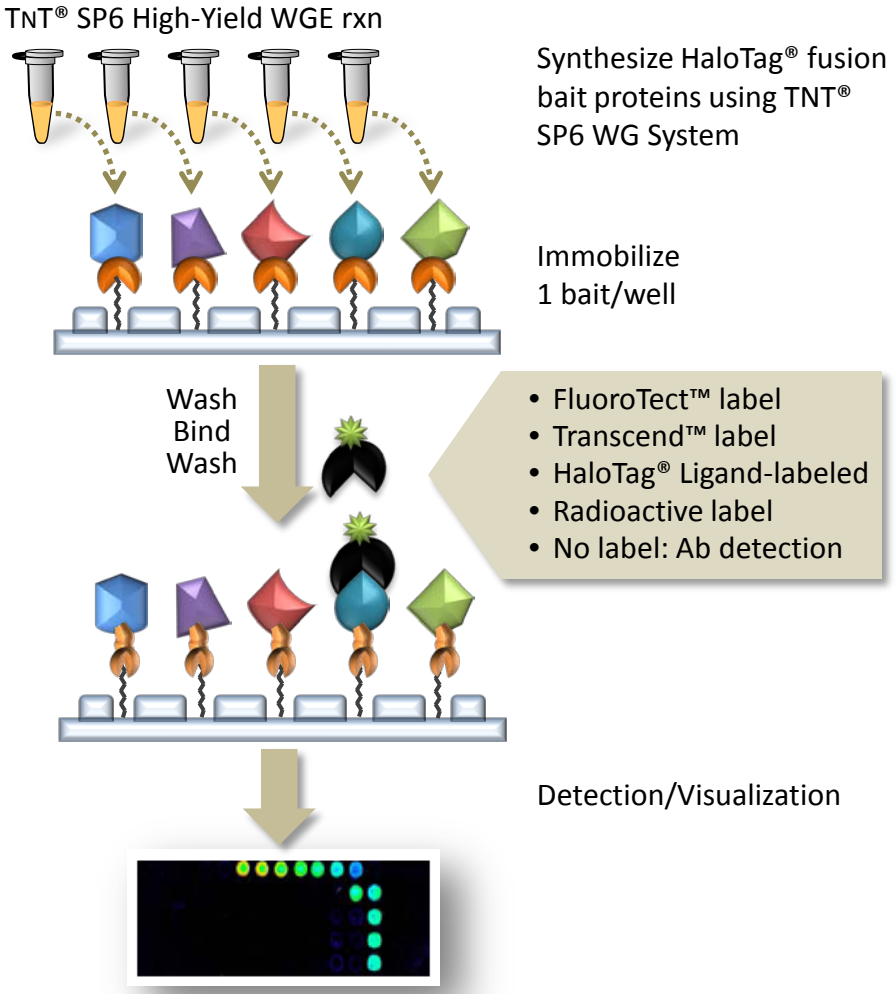
HaloLink™ Array Slide

- Immobilize ≤50 proteins per slide
- HaloTag® fusion dictates orientation
- Easy generation/customization of bait proteins using cell-free expression

Hurst, R., *et al.* (2009) *Analytical Biochemistry* **392**, 45-53.



# *Applying In Vitro (Cell-Free) Expression & HaloTag® Fusions to the Study Protein:Protein Interactions*

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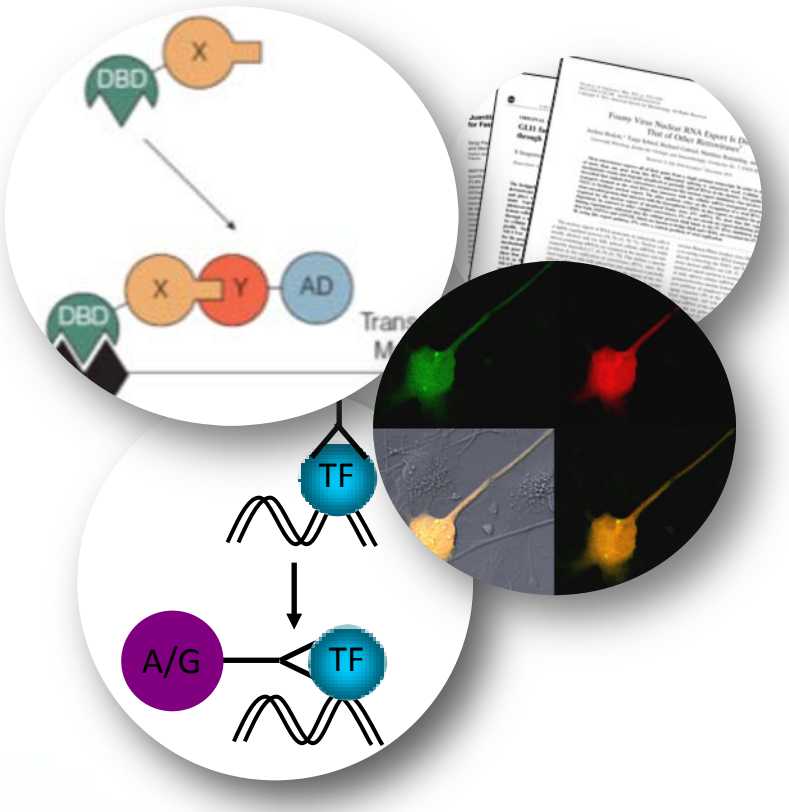
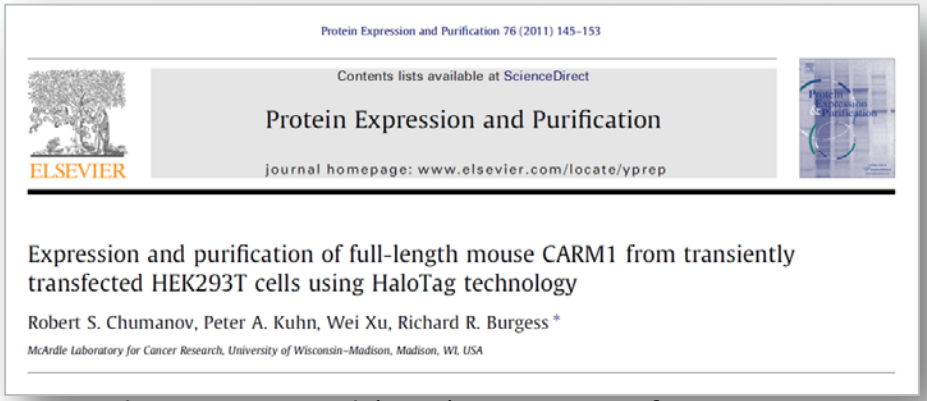
# ***Discovery Studies***
## *Identification of New In Vivo Protein Partners*




- Protein affinity purification
  - All in vivo
  - In vitro with purified bait + cellular extracts
- Yeast Two-hybrid assays
  - Library screening
- etc.

# *Case Study: Identification of New CARM1 Interacting Proteins/Substrates*




Chumanov, R., *et al.* (2011) *Prot. Exp. Purif.* **76**, 145-53

- **CARM1** (PRMT4)
  - Protein arginine methyl transferase
  - Transfers methyl group from SAM to Arg residues
  - Coactivator of transcriptional activation
- CARM1 studies have been hampered by inability to purify full-length protein
- **Goal**: Identify target substrates of CARM1 using affinity purification of interacting proteins followed by in vitro methylation experiments

# *Case Study: Identification of New CARM1 Interacting Proteins/Substrates*

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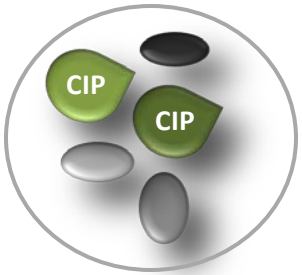
## Experimental Design – In Vitro Protein Affinity Purification

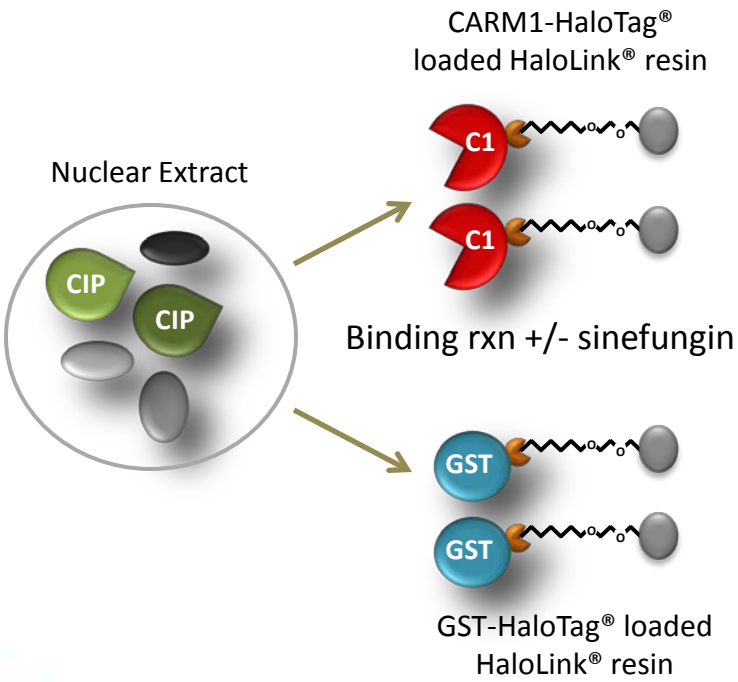
Nuclear Extract




**C1** = CARM1
**CIP** = CARM1 Interacting Protein

# Case Study: Identification of New CARM1 Interacting Proteins/Substrates

## Experimental Design – In Vitro Protein Affinity Purification

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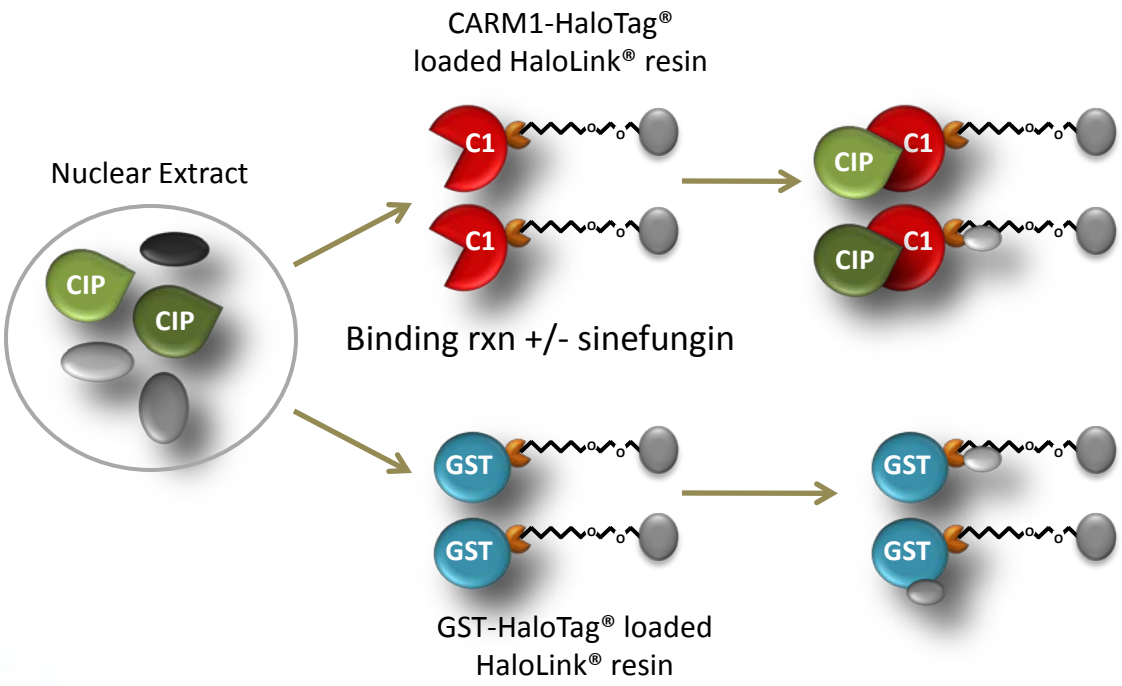
# Case Study: Identification of New CARM1 Interacting Proteins/Substrates

## Experimental Design – In Vitro Protein Affinity Purification

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# Case Study: Identification of New CARM1 Interacting Proteins/Substrates

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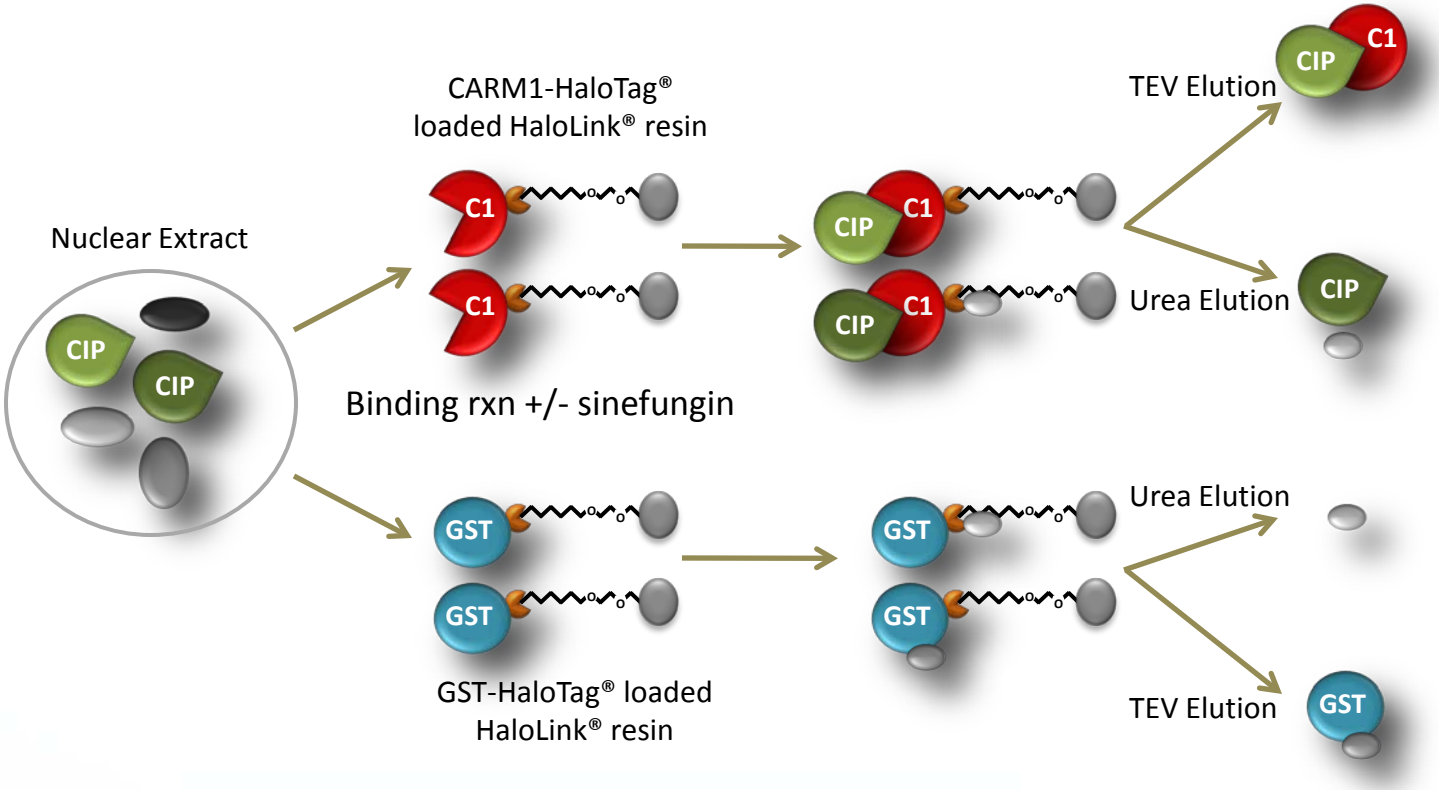
## Experimental Design – In Vitro Protein Affinity Purification

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# Case Study: Identification of New CARM1 Interacting Proteins/Substrates

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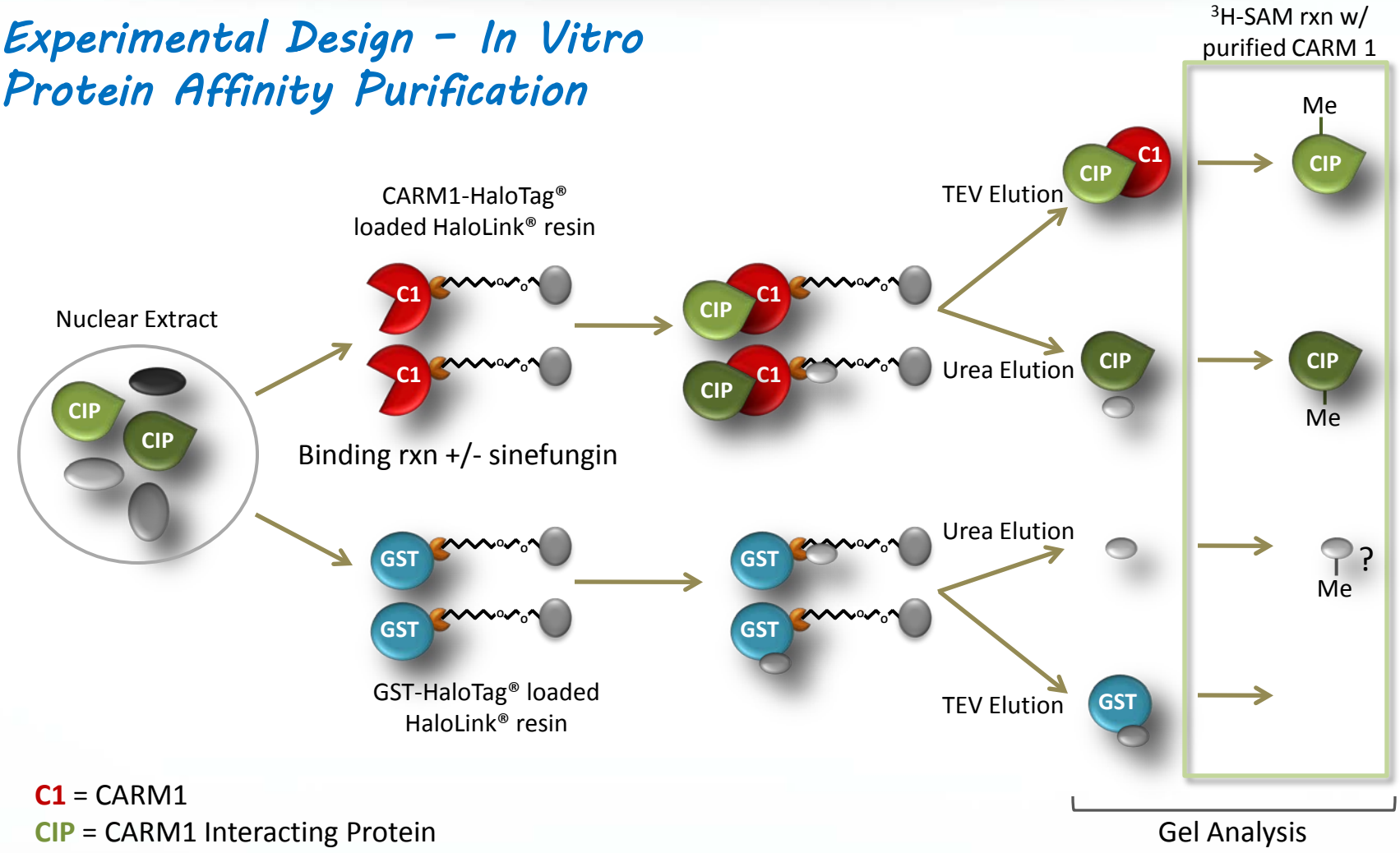


**C1** = CARM1

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# *Case Study: Identification of New CARM1 Interacting Proteins/Substrates*

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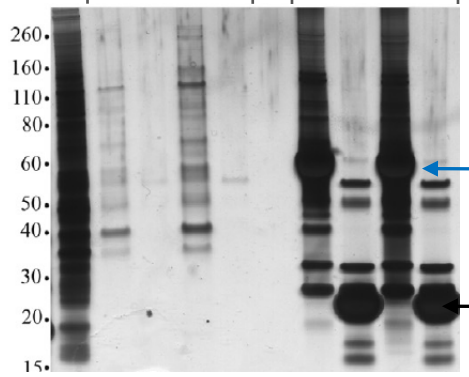
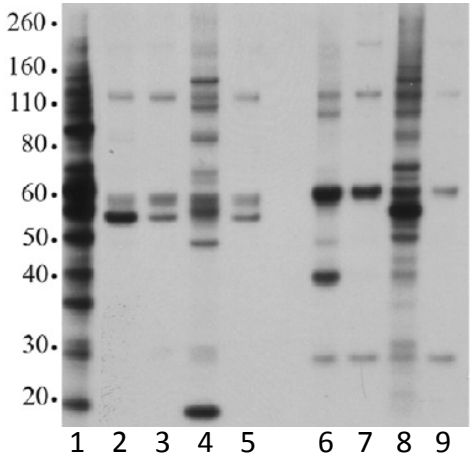
| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| HaloTag®-CARM1 | | + | | + | | | + | | + | |
| HaloTag®-GST Control | | | + | | + | | | + | | + |
| Nuclear Lysate Control | + | | | | | | | | | |
| Sinefungin Active Site Inhibitor | | | | + | + | | | | + | + |
| | | Urea Elution | | | | | TEV Elution | | | |

Silver Stained Protein Gel



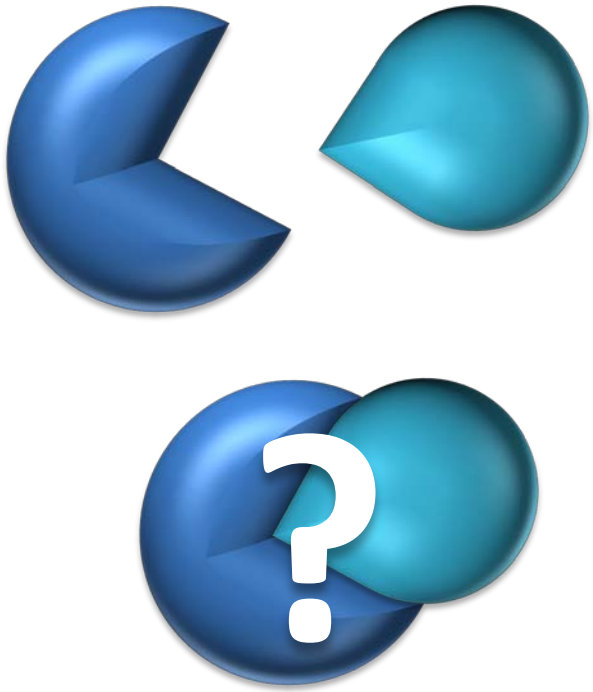

3H-Methylation Assay
(CARM1 Substrates)




## Results

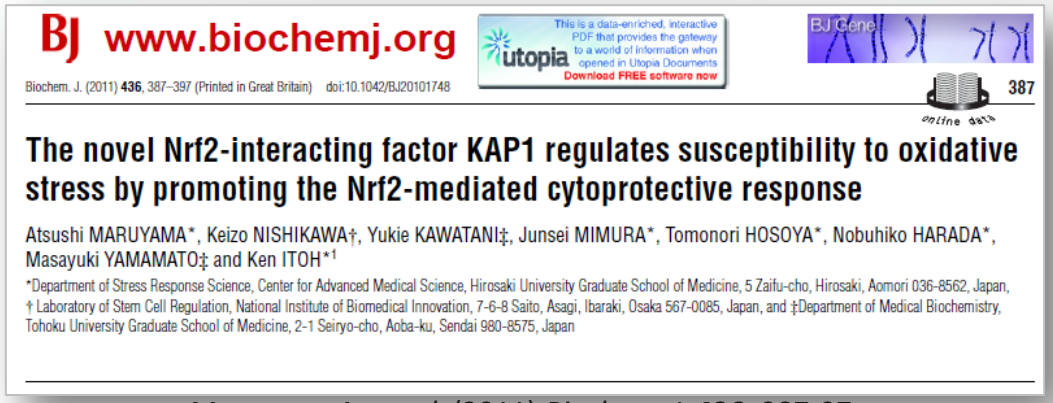
1. Identified multiple CARM1 Interacting Proteins
2. Many CIPs are also CARM1 substrates
3. Sinefungin increases capture efficiency of CIPs/substrates

# ***Verification Studies***
## *Confirming Two (or more) Proteins Interact*




- In Vitro Confirmation
  - Protein affinity purification (pulldown assays)
  - Co-Immunoprecipitations
- In Vivo (cells) Confirmation
  - Protein affinity purification (pulldown assays)
  - Co-immunoprecipitations
  - Mammalian two-hybrid assays

# *Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction*



BJ www.biochemj.org



Biochem. J. (2011) **436**, 387–397 (Printed in Great Britain) doi:10.1042/BJ20101748



**The novel Nrf2-interacting factor KAP1 regulates susceptibility to oxidative stress by promoting the Nrf2-mediated cytoprotective response**

Atsushi MARUYAMA*, Keizo NISHIKAWA†, Yukie KAWATANI‡, Junsei MIMURA*, Tomonori HOSOYA*, Nobuhiko HARADA*, Masayuki YAMAMATO‡ and Ken ITOH*[1]

*Department of Stress Response Science, Center for Advanced Medical Science, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan, † Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, 7-6-8 Saito, Asagi, Ibaraki, Osaka 567-0085, Japan, and ‡Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan

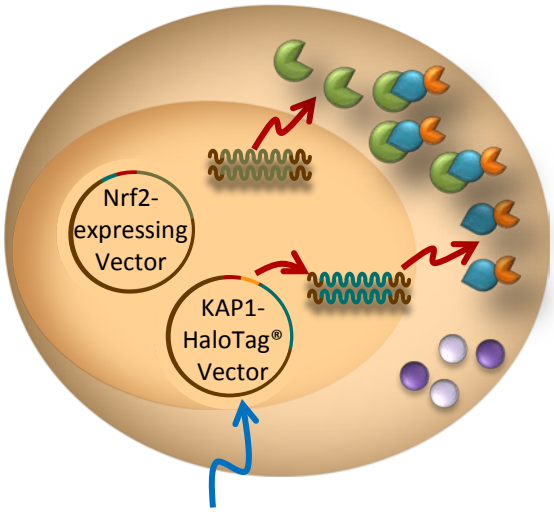
Maruyama A., *et al.* (2011) *Biochem. J.* **436**, 387-97

- **Nrf2** (Nuclear factor-erythroid 2-related factor)
  - Transcription factor
  - Co-ordinately regulates ARE (antioxidant-response element)-mediated induction of cytoprotective genes in response to oxidative stress & electrophiles
- Mechanism of action is not well understood
- **Goal**: After identification of KAP1 as an Nrf2 interacting protein, confirm and characterize the interaction

# Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction

## Experimental Design – Confirming the Interaction with a HaloTag® Pulldown Assay

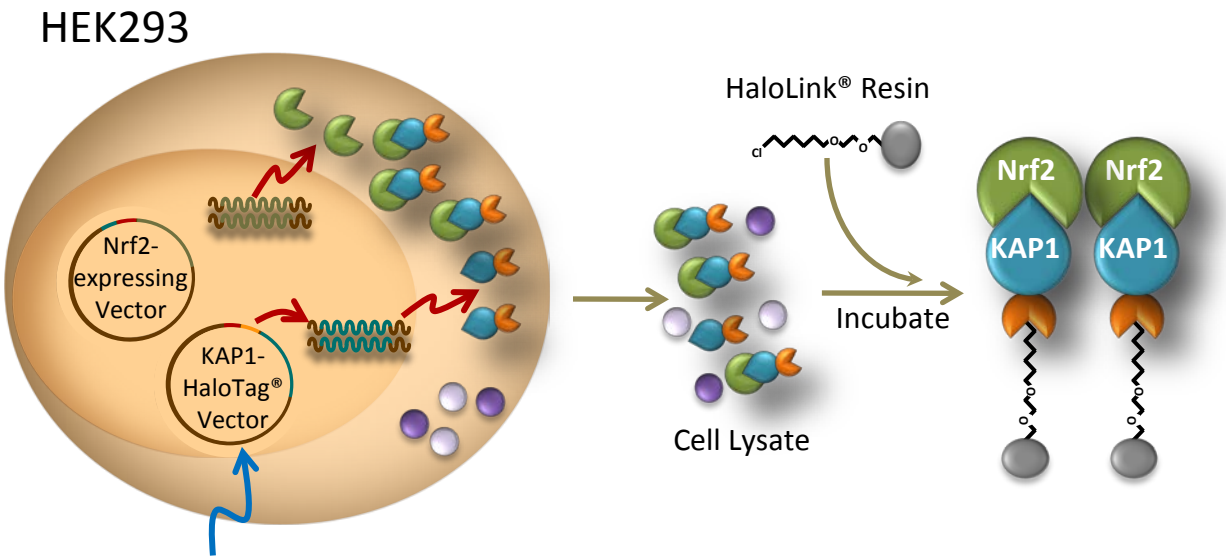
HEK293




Validated HaloTag® clones available from www.promega.com/kazusa
A partnership between Kazusa DNA Research Institute and Promega.

# Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction

## Experimental Design – Confirming the Interaction with a HaloTag® Pulldown Assay




Validated HaloTag® clones available from www.promega.com/kazusa
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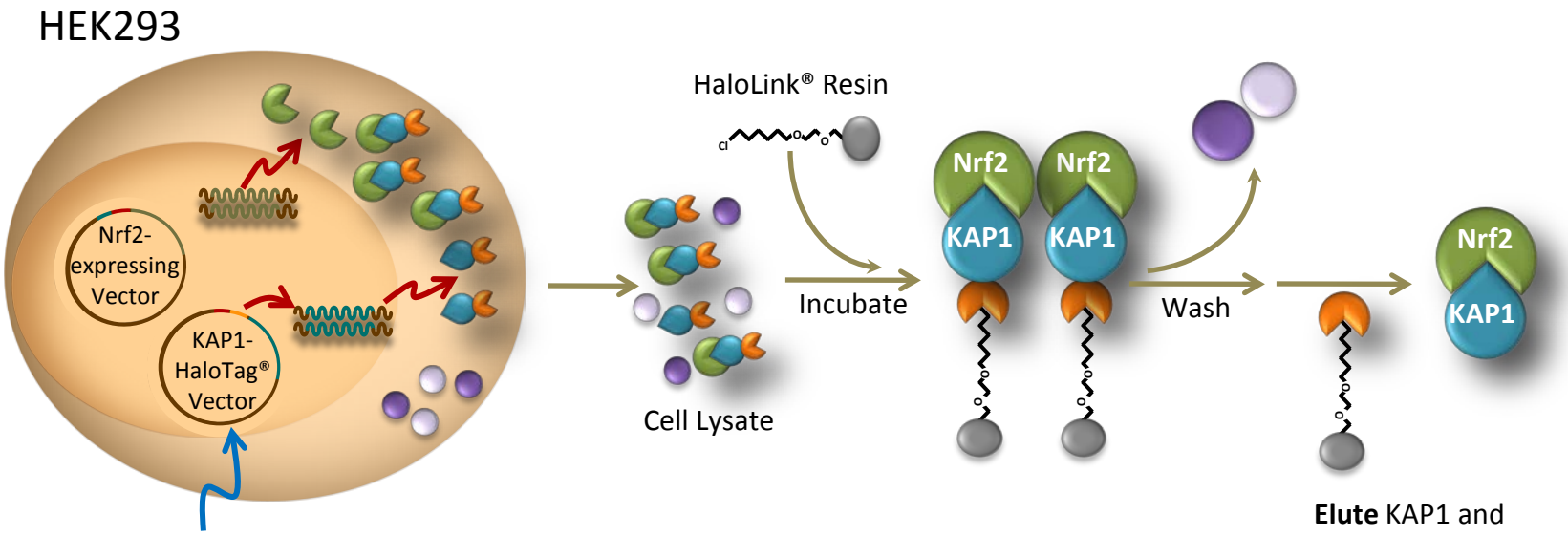
# Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction

## Experimental Design – Confirming the Interaction with a HaloTag® Pulldown Assay

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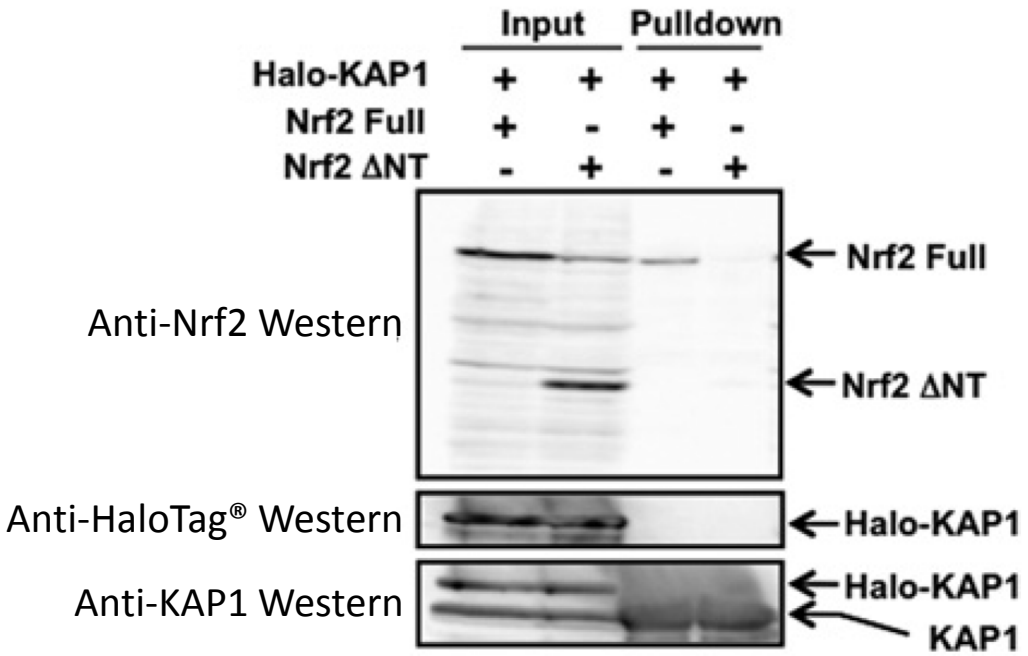
# *Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction*




Data from Maruyama A., *et al.* (2011) *Biochem. J.* **436**, 387-97

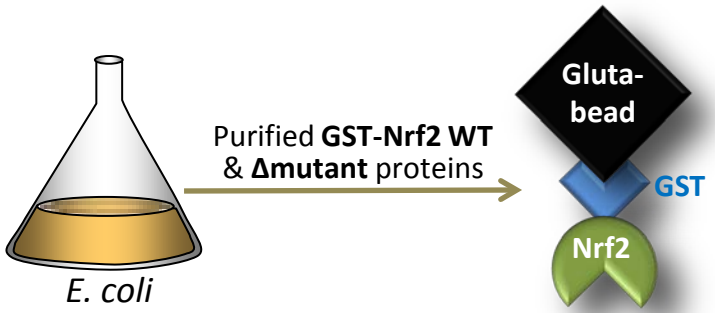
## Results

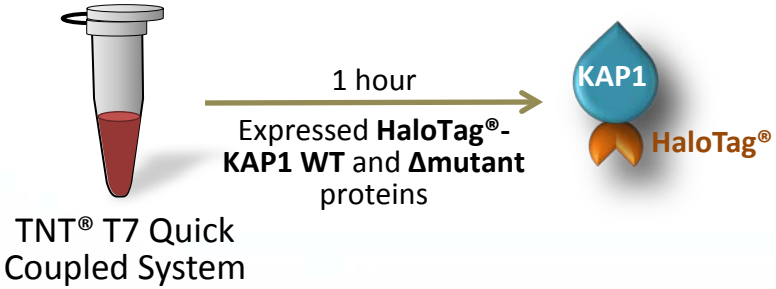
1. Verified KAP1:Nrf2 interaction in vivo

2. N-terminus of Nrf2 is required for interaction with KAP1

# Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction

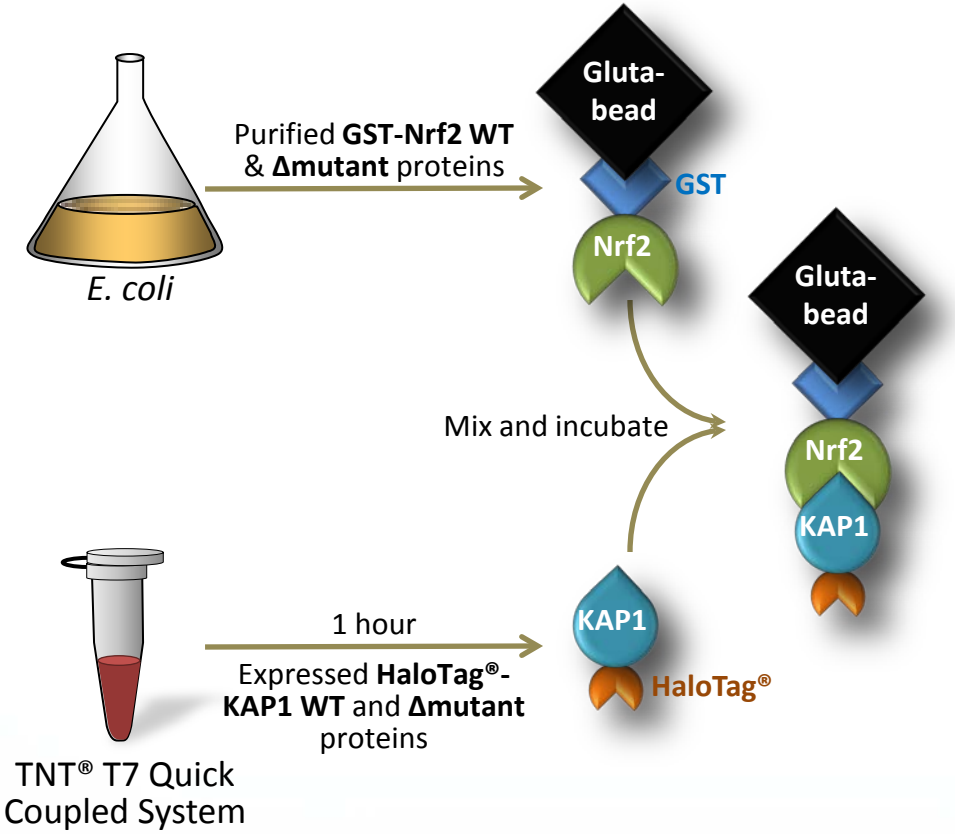
## Experimental Design – Characterizing the Interaction with GST Pulldowns



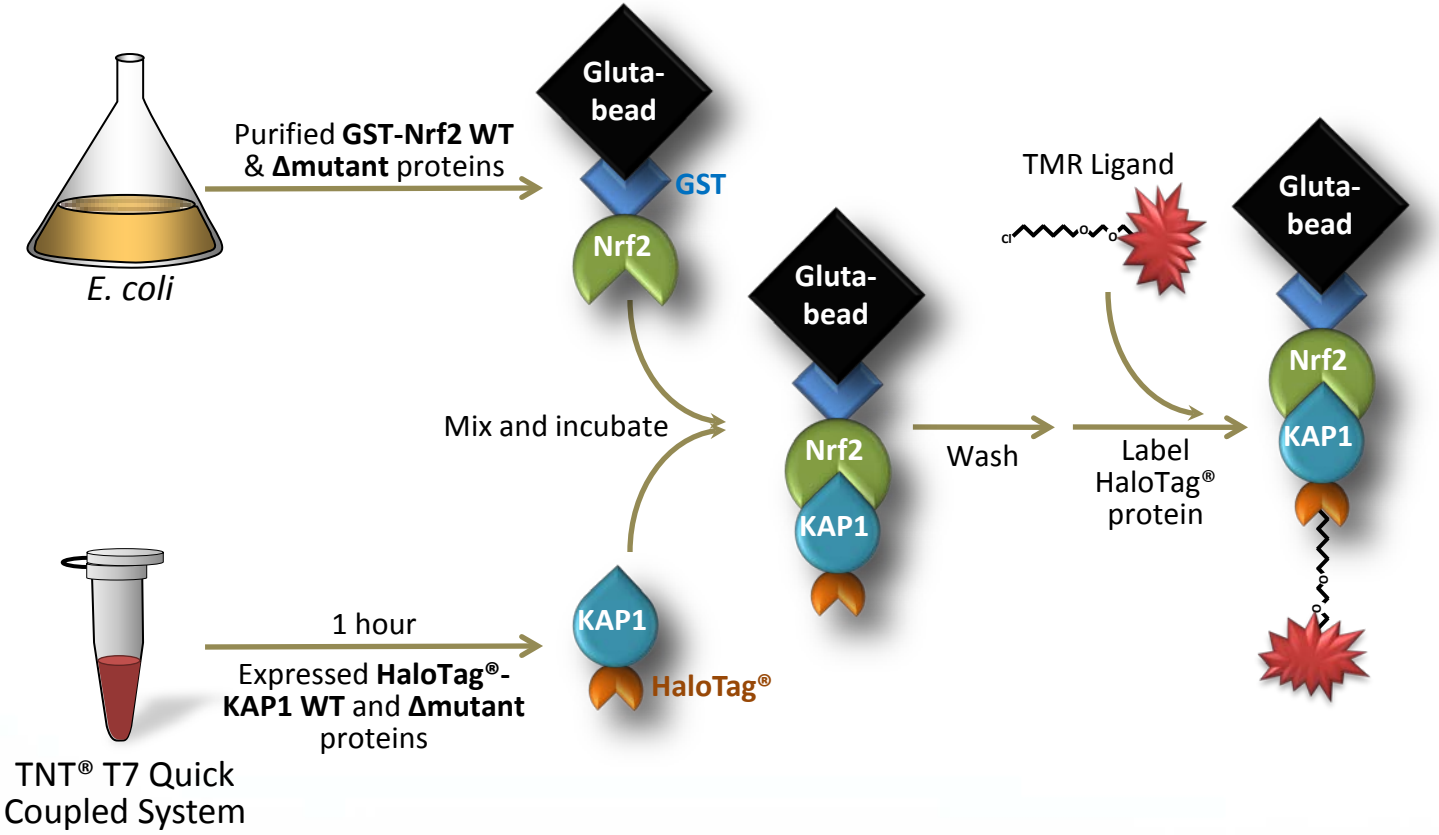
# Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction

## Experimental Design – Characterizing the Interaction with GST Pulldowns



# Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction

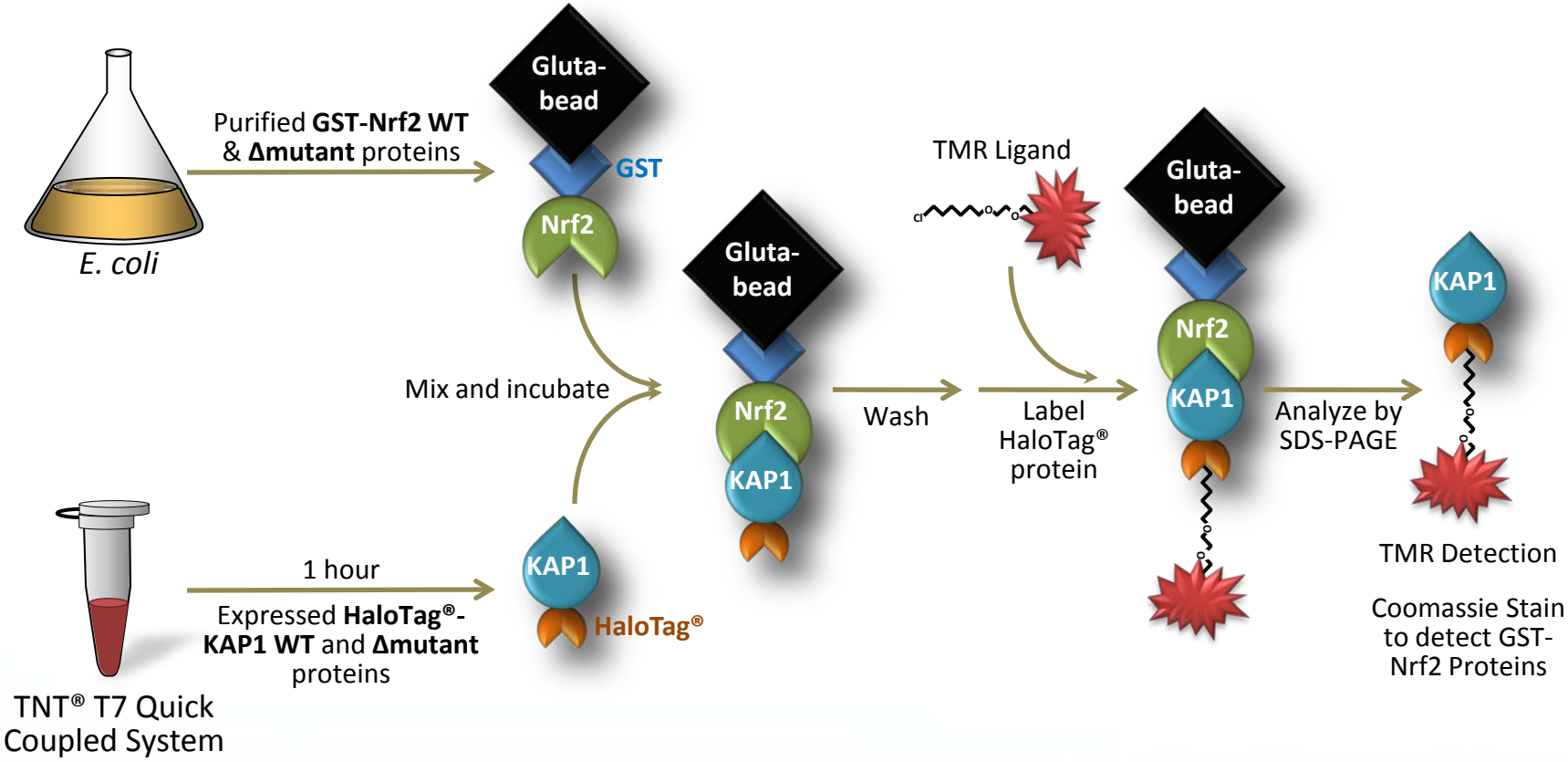
## Experimental Design – Characterizing the Interaction with GST Pulldowns



# Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction

Promega

## Experimental Design – Characterizing the Interaction with GST Pulldowns



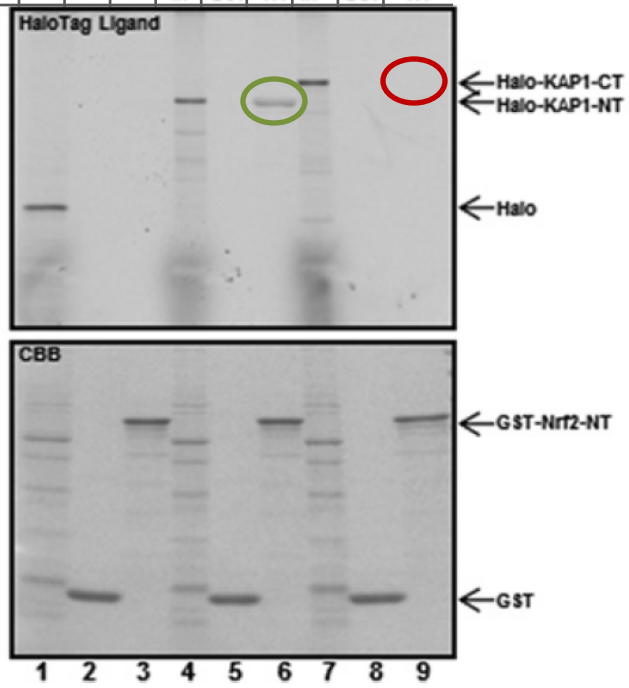
# *Case Study: Verification & Characterization of a Novel Nrf2:KAP1 Interaction*

Promega

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| HaloTag® Control | IN | + | + | | | | | | |
| **HaloTag®-KAP1 NT** | | | | **IN** | **+** | **+** | | | |
| **HaloTag®-KAP1 CT** | | | | | | | **IN** | **+** | **+** |
| GST | | + | | | + | | | + | |
| GST-Nrf2 NT | | | + | | | + | | | + |

TMR Detection (HaloTag®-KAP1) (Fluoroimager)

Coomassie Stained Gel (GST, GST-Nrf2)



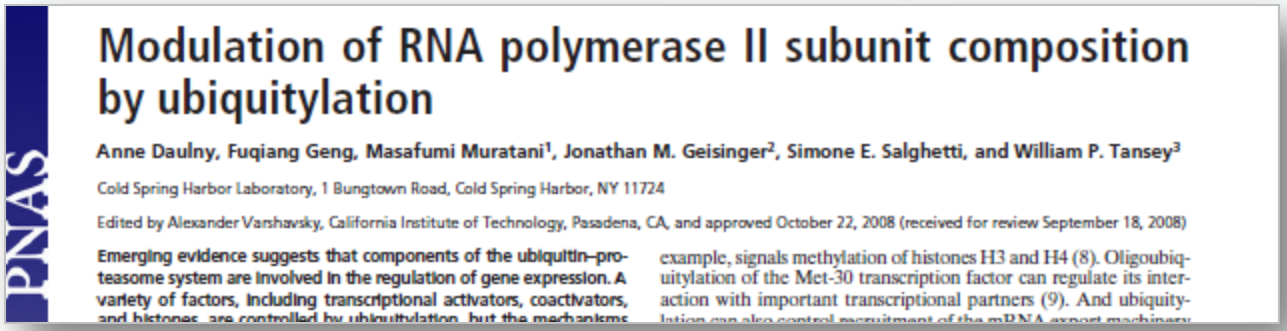

Data from Maruyama A., *et al.* (2011) *Biochem. J.* **436**, 387-97

## Results

1. N-terminus (NT) of KAP1 promotes the interaction with Nrf2

2. Further analysis showed that activation domain within Nrf2 NT is responsible for interaction

# *Case Study: Testing for Direct Interaction of Asr1 with the RNAP II CTD*



PNAS

## Modulation of RNA polymerase II subunit composition by ubiquitylation

Anne Daulny, Fuqiang Geng, Masafumi Muratani[1], Jonathan M. Geisinger[2], Simone E. Salghetti, and William P. Tansey[3]

Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724

Edited by Alexander Varshavsky, California Institute of Technology, Pasadena, CA, and approved October 22, 2008 (received for review September 18, 2008)

**Emerging evidence suggests that components of the ubiquitin–proteasome system are involved in the regulation of gene expression. A variety of factors, including transcriptional activators, coactivators, and histones, are controlled by ubiquitylation, but the mechanisms**

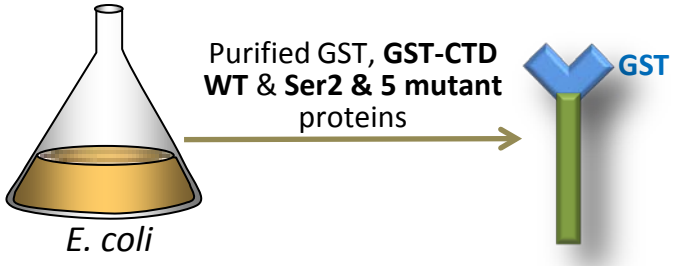
example, signals methylation of histones H3 and H4 (8). Oligoubiquitylation of the Met-30 transcription factor can regulate its interaction with important transcriptional partners (9). And ubiquitylation can also control recruitment of the mRNA export machinery

Daulny A., *et al.* (2008) *Proc. Nat. Acad. Sci.* **105**, 19649-54

- **Asr1**
  - Involved in the yeast alcohol stress response
  - Two-hybrid data on a mammalian protein with limited Asr1 homology suggested a possible role of Asr1 in ubiquitylation of RNAP II in yeast.
- Interested in understanding if Asr1 does indeed play a role in ubiquitylation of RNAP II and initial experiments in the paper demonstrated Asr is a ubiquitin-ligase.
- **Goal**: Demonstrate direct binding of Asr1 to the carboxy terminal domain (CTD) of RNAP II and characterize the interaction based on phosphorylation of the CTD.

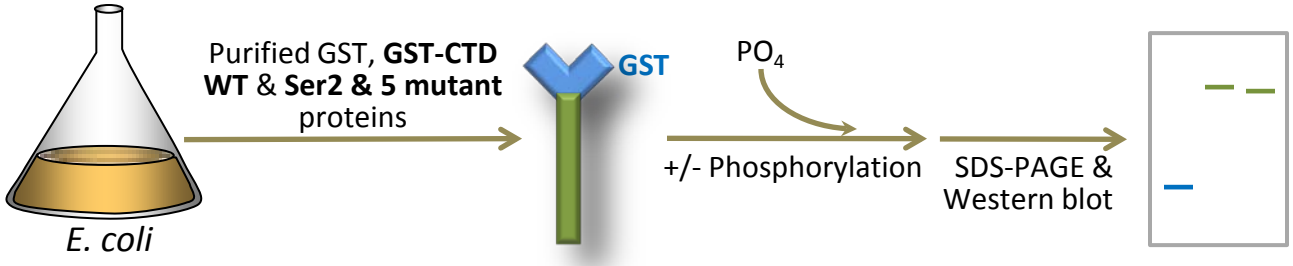
# Case Study: Testing for Direct Interaction of Asr1 with the RNAP II CTD

## Experimental Design – Use Far Westerns to Detect Interactions



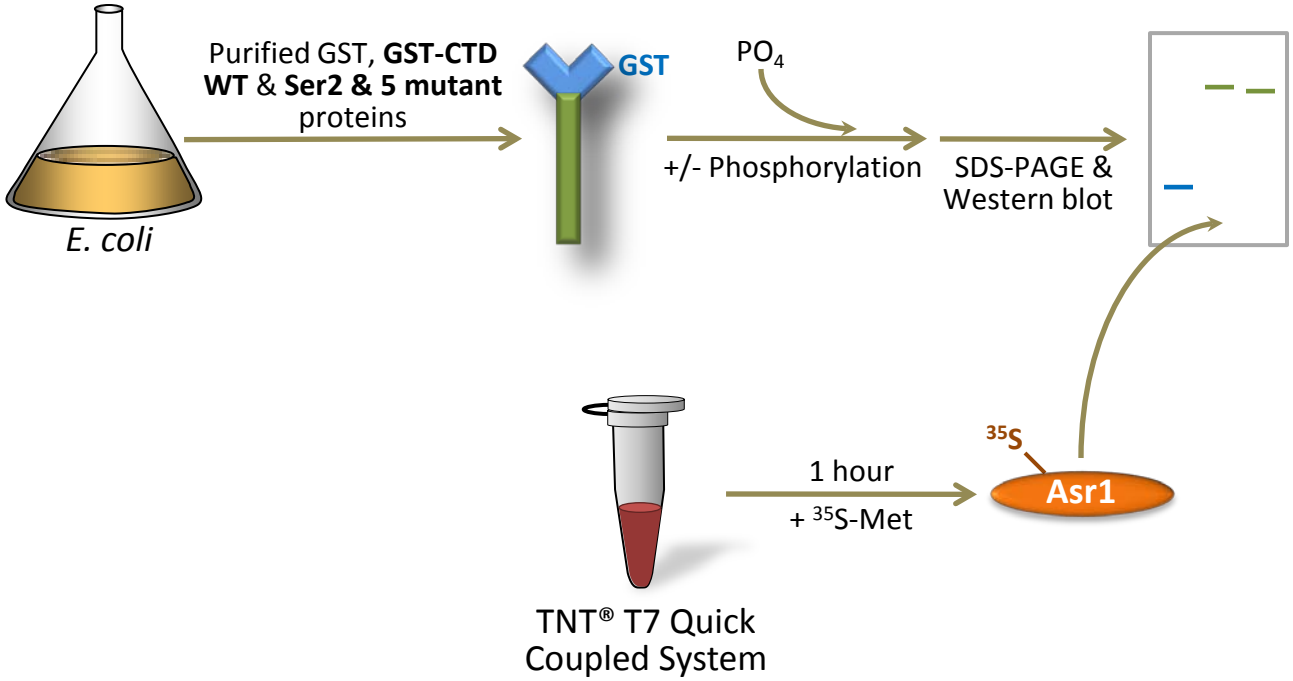
# Case Study: Testing for Direct Interaction of Asr1 with the RNAP II CTD

## Experimental Design – Use Far Westerns to Detect Interactions



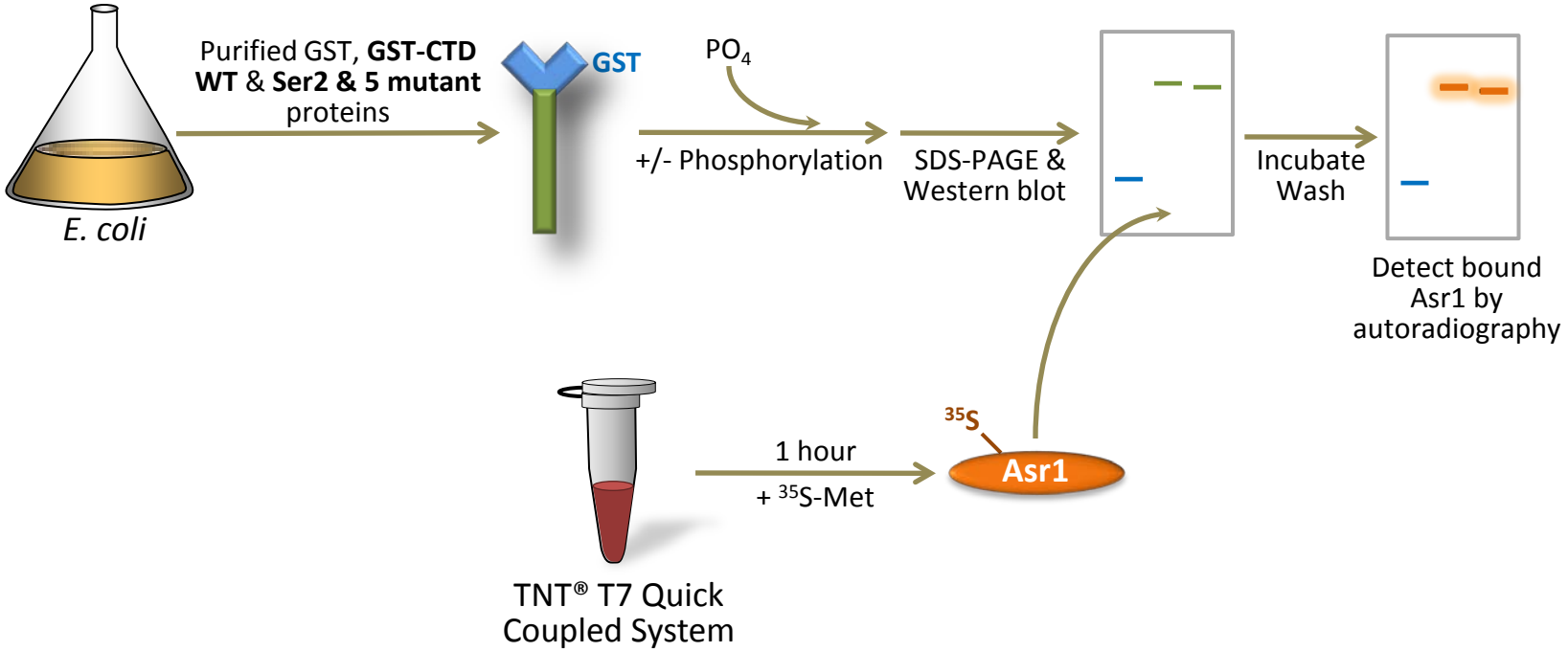
# Case Study: Testing for Direct Interaction of Asr1 with the RNAP II CTD

## Experimental Design – Use Far Westerns to Detect Interactions

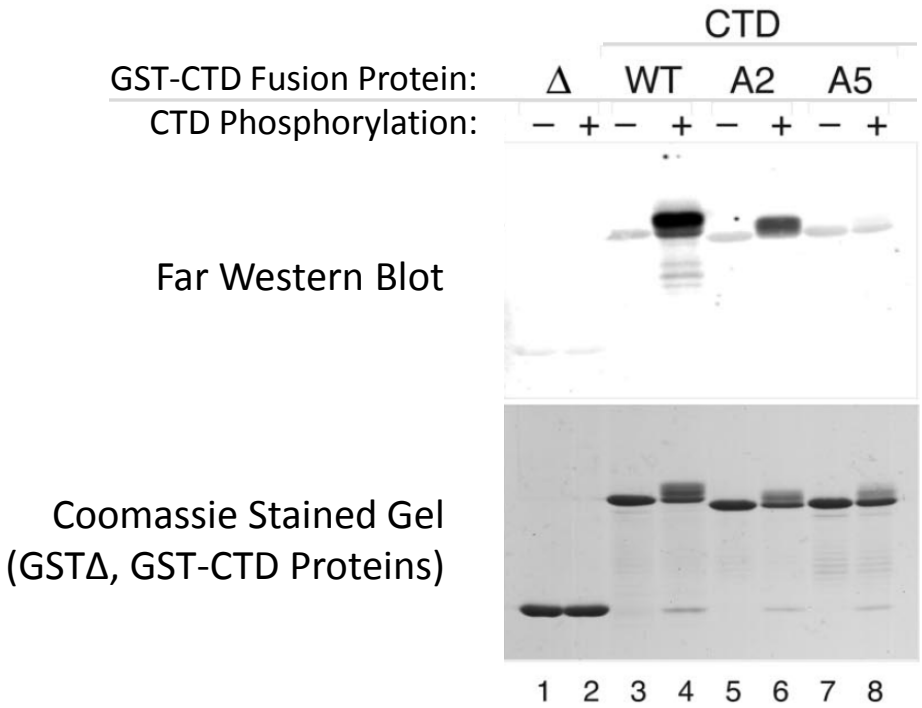


# Case Study: Testing for Direct Interaction of Asr1 with the RNAP II CTD

## Experimental Design – Use Far Westerns to Detect Interactions



# *Case Study: Testing for Direct Interaction of Asr1 with the RNAP II CTD*




**A2** = Ser2 to Ala mutation
**A5** = Ser5 to Ala mutation

Data from Daulny A., *et al.* (2008) *Proc. Nat. Acad. Sci.* **105**, 19649-54

## Results

1. Asr1 binds directly to the RNAP II CTD in a phosphorylation dependent manner.
2. Mutation of Ser5 to alanine prevents phosphorylation at Ala5 & blocks Asr1 binding.
3. Phosphorylation of Ser2 plays less of a role in promoting Asr1 binding.

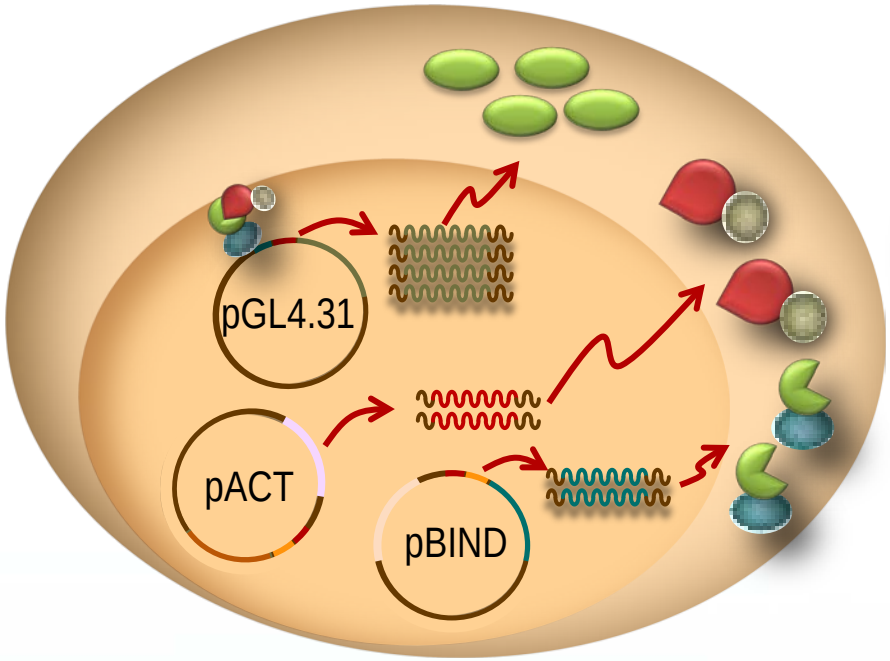
# *Mammalian Two-Hybrid Assays*

Adapting the Yeast Two-Hybrid Assay to Mammalian Cells

Promega

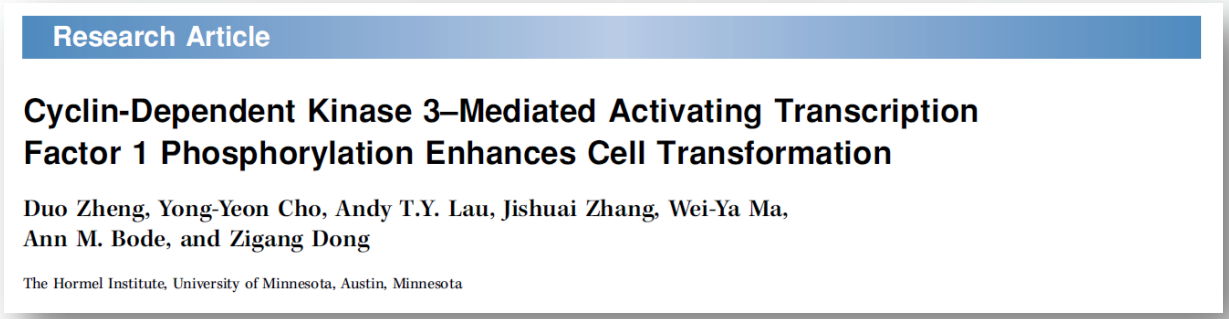
# *Adaptation of the Yeast Two Hybrid Assay to Mammalian Cells: CheckMate™*


1. TRANSFECT
2. CULTURE 2-3 DAYS
3. DUAL-LUCIFERASE ASSAY




15 citations in 2011 Jan-July

# *Case Study: Testing for Interaction of Cdk3 with ATF1 in Mammalian Cells*



Research Article

**Cyclin-Dependent Kinase 3–Mediated Activating Transcription Factor 1 Phosphorylation Enhances Cell Transformation**

Duo Zheng, Yong-Yeon Cho, Andy T.Y. Lau, Jishuai Zhang, Wei-Ya Ma, Ann M. Bode, and Zigang Dong

The Hormel Institute, University of Minnesota, Austin, Minnesota

Zheng, D., *et al.* (2008) *Cancer Res.* **68**, 7650-60.

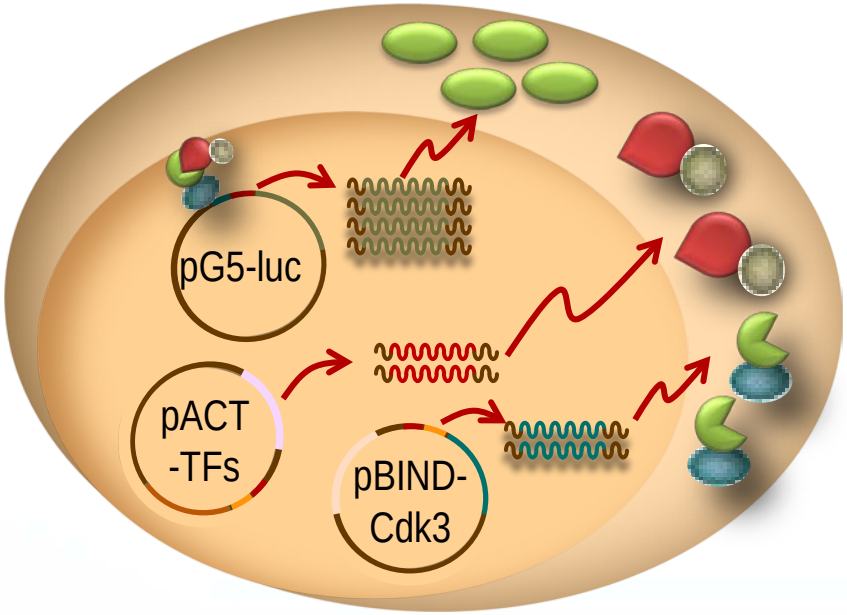
- **Cdk3** (Cyclin-dependent kinase 3)
  - Ser/Thr kinase
  - Highly expressed in glioblastoma tissues and cell lines
- Interested in determining if Cdk3 is interacting with and phosphorylating transcriptional regulatory proteins in glioblastoma cells to upregulate transcription
- **Goal**: Screen various transcription factors (TFs) using the CheckMate Mammalian Two Hybrid Assay to determine if they interact with Cdk3 in vivo.

# *Case Study: Testing for Interaction of Cdk3 with ATF1 in Mammalian Cells*

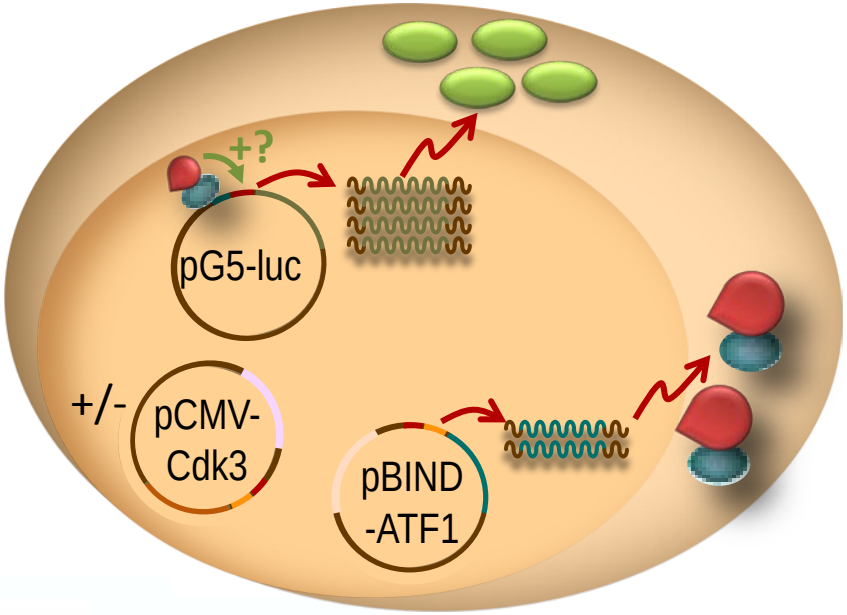
Promega

## *Experimental Design*

CheckMate™ Two Hybrid Assay



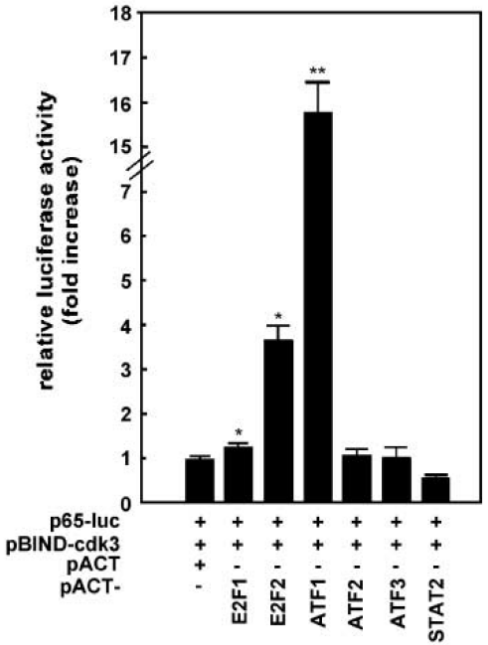

Modified CheckMate™ One Hybrid Assay



# Case Study: Testing for Interaction of Cdk3 with ATF1 in Mammalian Cells

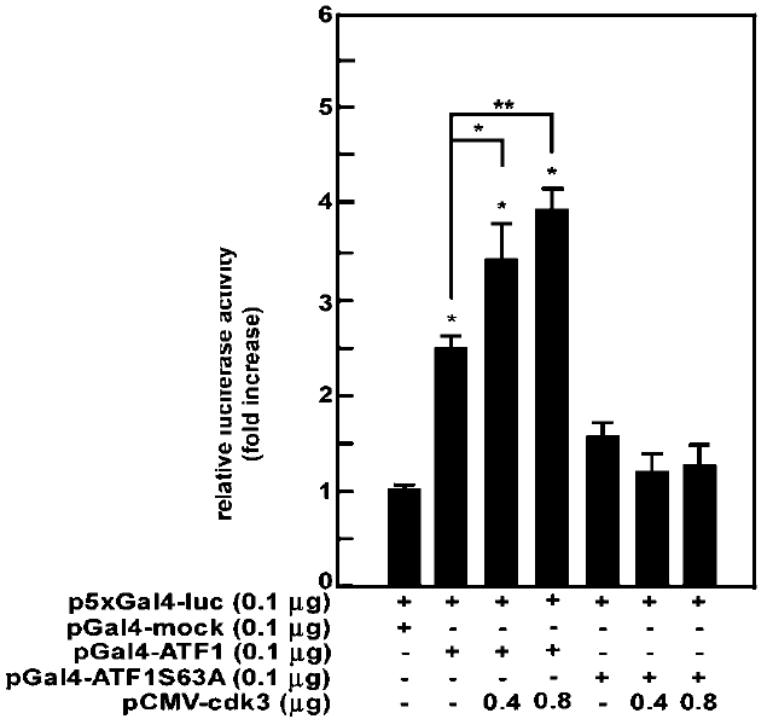
Promega

## Two-Hybrid Assay




Identified ATF1 as a new binding partner for Cdk3 (E2F2 = positive control)

## One-Hybrid Assay




Phosphorylation of ATF1 increases transcriptional activation activity

Data from Zheng, D., *et al.* (2008) *Cancer Res.* **68**, 7650-60.

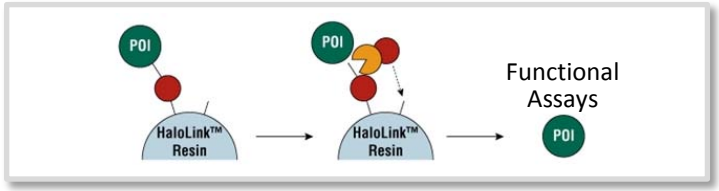
# *Additional Applications of In Vitro Expression*

Promega

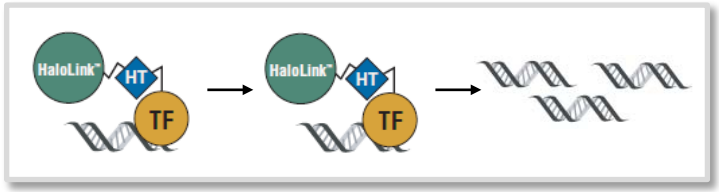
- Functional Assays
  - Enzymatic activity assays
    - Screening applications
- Post-translational modification analysis
- In Vitro Protein-DNA & Protein-RNA Interactions Studies
  - Gel-shift assays

# *Additional Applications HaloTag® Technology*

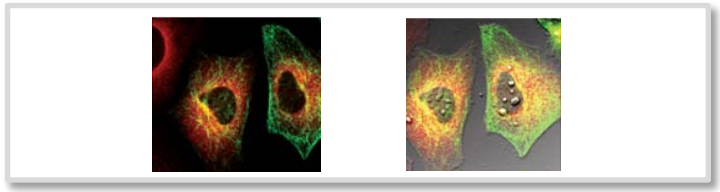
- HaloTag® Technology
  - Functional assays with protein purified from *E. coli* and mammalian cells




  - Chromatin pull-down assays with HaloCHIP™ System




  - Protein localization, trafficking and turnover



  - Target protein imaging in whole animals

## *Summary*

- In vitro (cell-free) expression provides a rapid means to produce full-length or deletions of your protein of interest for interaction studies.
- HaloTag® Fusions provide a multifunctional handle on your protein of interest to study protein interactions both in vitro and in vivo.
- The combination of in vitro (cell-free) expression and HaloTag® fusions is a powerful tool for protein interaction studies.
- The CheckMate™ Mammalian Two-Hybrid System is another tool for studying protein interactions in mammalian cells.

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*Your Local Promega Resource*

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- Most responses within 2hrs

North American Branch Technical Support Team