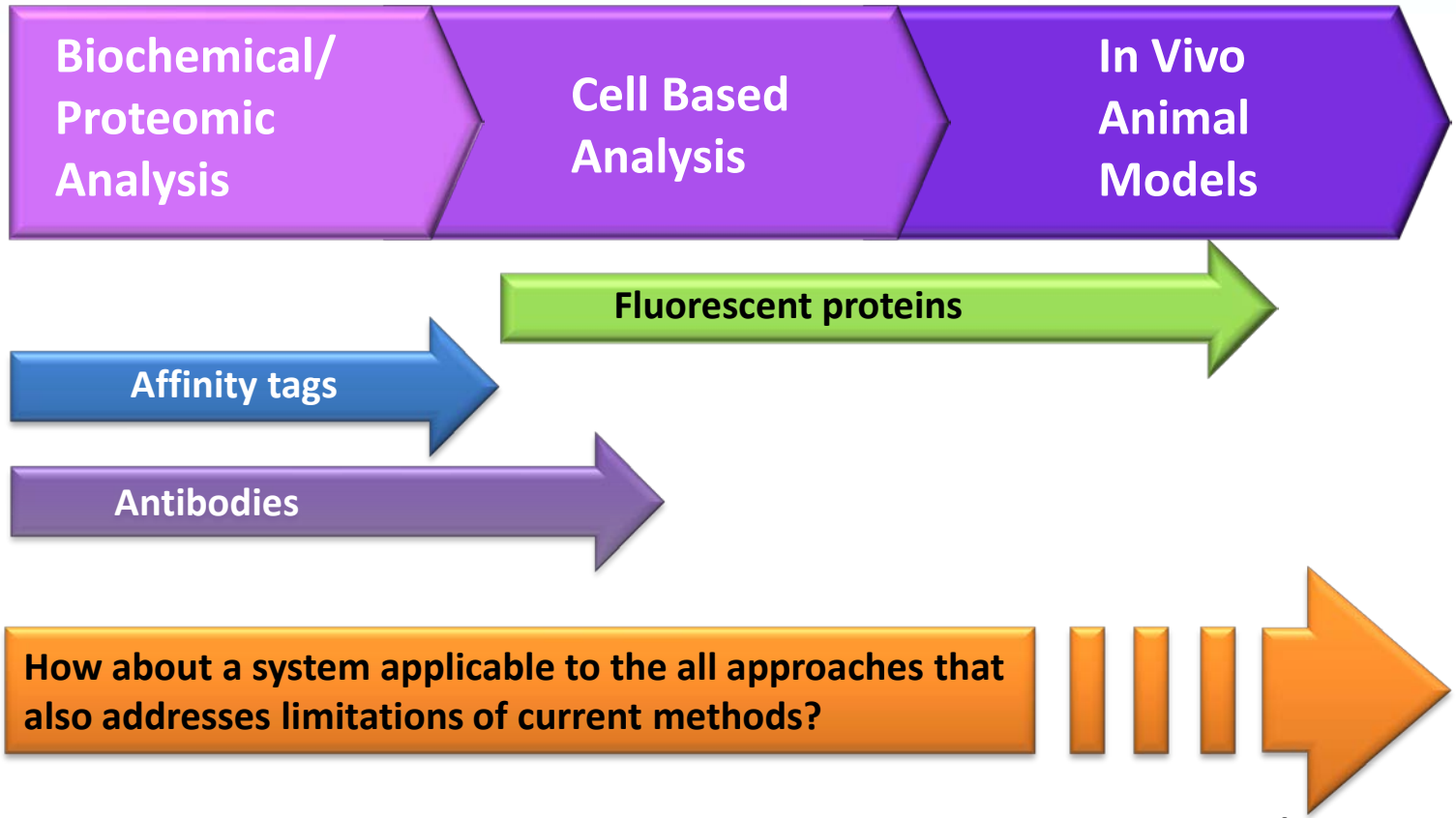
Overcoming Challenges of Protein Analysis in Mammalian Systems

Danette L. Daniels, Ph.D.

Promega

Current Technologies for Protein Analysis



- Minimal interference with protein of interest
- Detection/real-time imaging
- High Signal/background
- Efficient capture/isolation
- Differential labeling

HaloTag Platform

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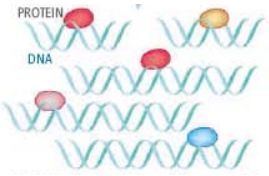
Biochemical/ Proteomic Analysis → **Cell Based Analysis** → ***In Vivo*** **Animal Models**

Protein purification
Protein arrays
Protein interactions

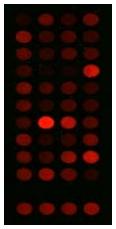
Protein localization
Real time imaging
Protein trafficking
Protein turnover

In vivo **fluorescent imaging**

HaloTag®
Purification

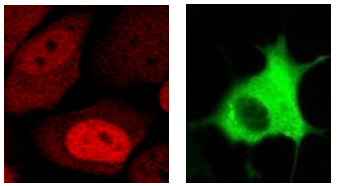


HaloCHIP™
Protein:DNA



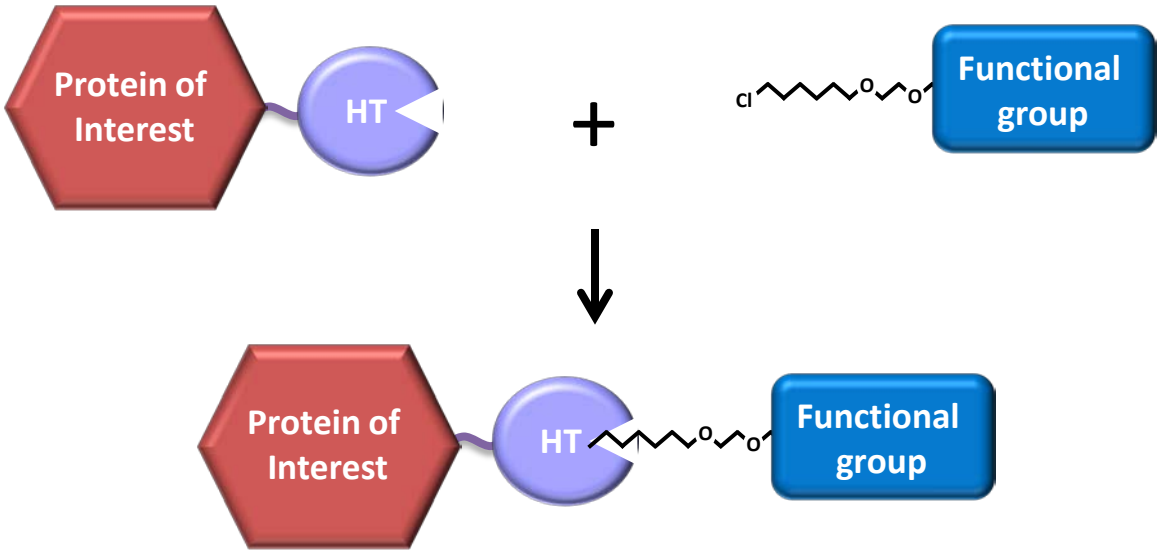
HaloLink™
Protein Arrays

HaloTag®
Pull-Down



Fluorescent
Ligands

HaloTag is a Genetically Engineered Protein Fusion Tag



- A monomeric , 34 kDa, modified bacterial dehalogenase genetically engineered to covalently bind specific, synthetic HaloTag® ligands
- Irreversible, covalent attachment of chemical functionalities
- Suitable as either N- or C- terminal fusion

Mutagenized HaloTag® Protein Enables Covalent HaloTag®-Ligand Complex

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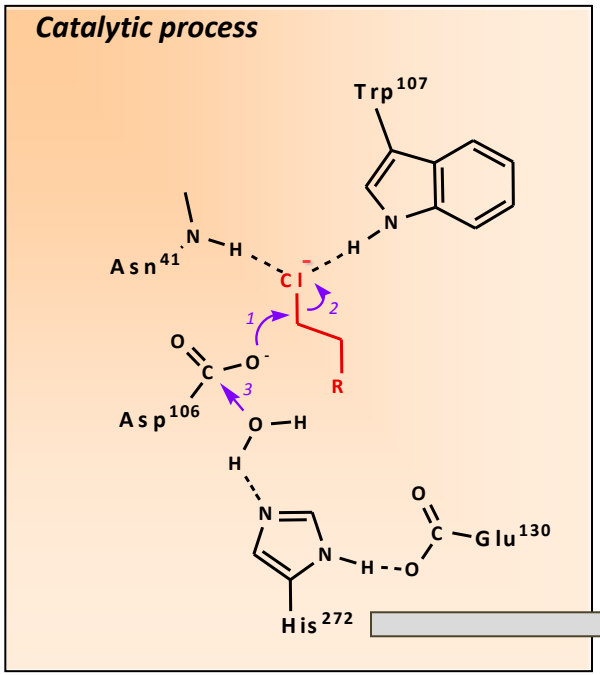
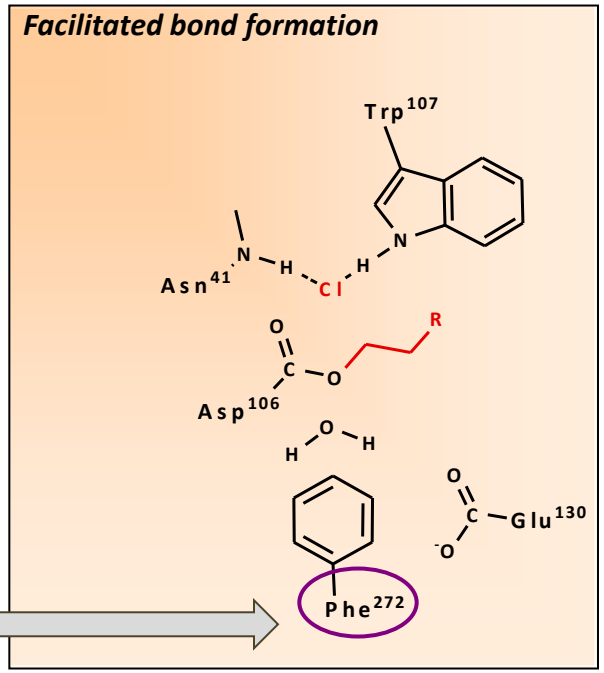
Hydrolase (DhaA)



HaloTag®



HaloTag®:

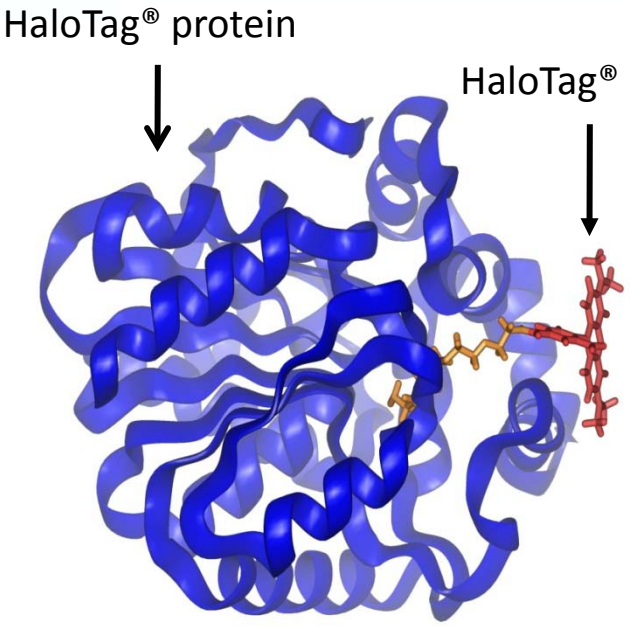
- 34kDa protein
- Monomeric
- Single change: His272Phe for covalent bond.

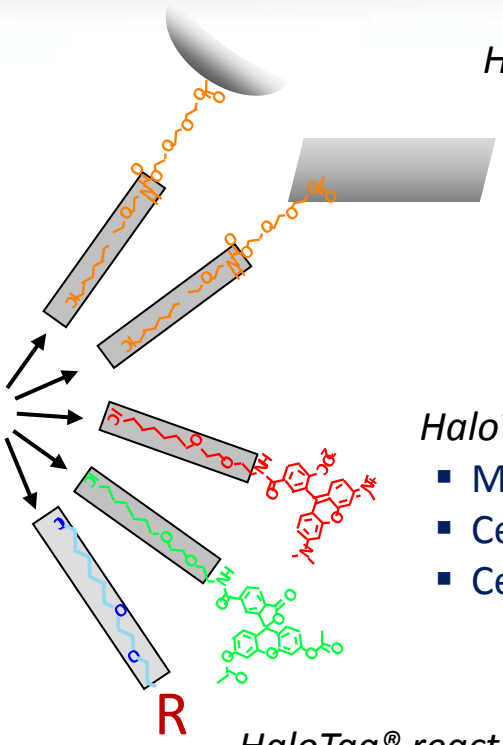
Covalent bond:

- Stable after denaturation.
- Confirmed by Mass spec.

- **DhaA is a rare, bacterial hydrolase.**
- **Binds to chloroalkane substrates.**
- **Forms a covalent intermediate.**
- **Activation of water by His drives hydrolysis.**

Ligands Impart Multi-functionality





HaloTag® surfaces

- Nonmagnetic resin
- Magnetic resin
- Glass slides

HaloTag® fluorescent ligands

- Many different colors
- Cell permeable ligands
- Cell non-permeable ligands

HaloTag® reactive ligands

- Attach functional group of choice

i.e. Quantum dots, PET ligands…

- ***Selectable functionalties*: a single fusion construct may be attached to a broad range of functional properties**

Generation of a N- & C-Terminal HaloTag® Fusions

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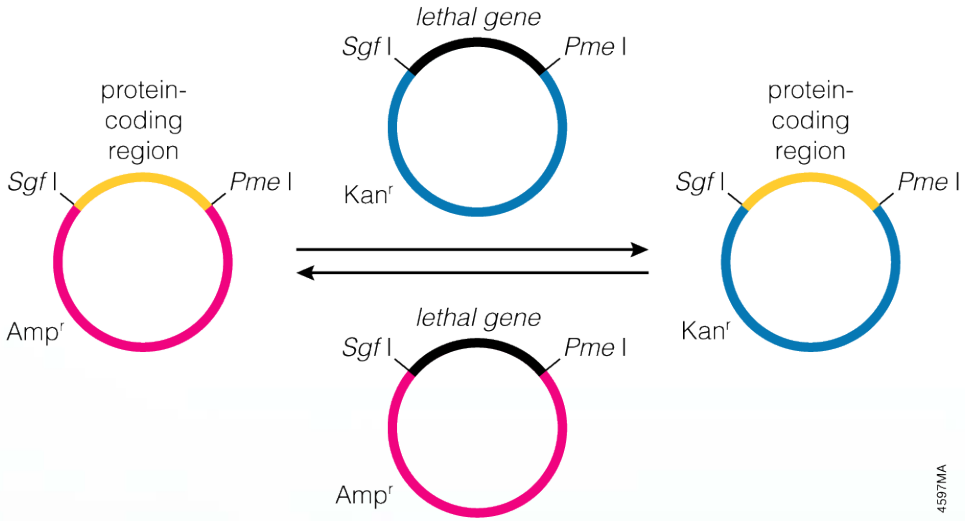
Flexi vectors for expression of HaloTag fusions in mammalian cells:

Expression	Promoter	N-terminal HaloTag®	C-terminal HaloTag®
Maximal expression	**CMV**	pFN21	pFC14
Promoter deletion series to optimize mammalian expression level	**CMVd1**	pFN22	pFC15
	CMVd2	pFN23	pFC16
	CMVd3	PFN24	pFC17

Flexi® Vector cloning system



- Flexible system for directional cloning that utilizes REs that are infrequent in ORFs
- Efficient transfer to multiple vectors
  Sequence once, transfer to many

No Cloning Necessary

HaloTag®-Fusion Clones are Readily Available

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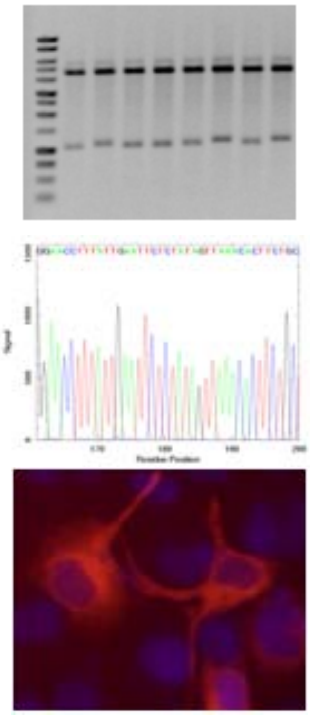
Kazusa DNA Research Institute

Human ORFs N-terminal fusion constructs in Flexi® vector pFN21A for expression in mammalian cells
http://www.kazusa.or.jp/kop/dsearch-e//

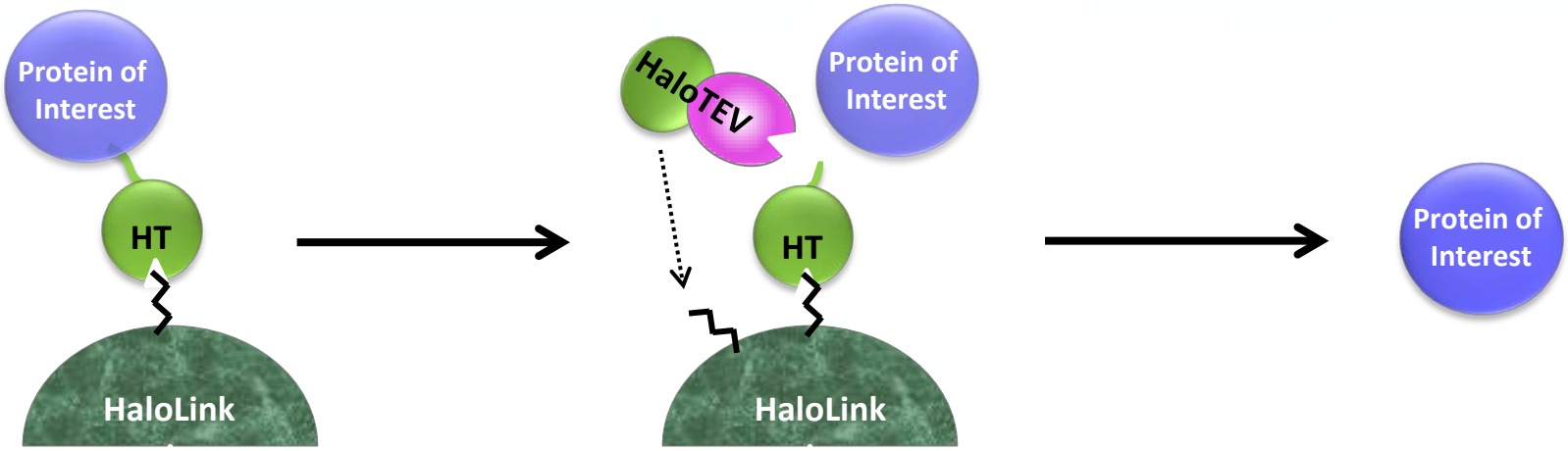
Features	Flexi-HaloTag Collection PID beginning with **FHC** as of Nov, 2010
Size of Collection	7,100 clones
Fusion Tag	HaloTag® *for protein purification, imaging & interactions*
Validated Clones	
> Sequence Validated	**Yes** (100% clones)
> Insert Validated	**Yes** (99.7% clones)
> Expression Validated	**Yes** (99.8% clones)
> Localization Validated	**Yes** (80.1% clones)
Format	DNA
Typical Delivery	2-4 weeks
Price ($USD)	$500.00 per clone



GeneCopoeia

Human and mouse ORFs N- /C- terminal fusion constructs available in OmicsLink™ vectors for expression in mammalian cells http://www.genecopoeia.com/product/halo/

HaloTag®-based Protein Purification Scheme



Covalent binding

Proteolytic release

POI proteolyticly released via HaloTEV

HaloTEV & HT remain permanently attached to HaloLink

Recovery

Protein free of tag

- **Covalent binding:**
  - Efficient capture regardless of expression level
  - Stringent washes possible
  - Minimal loss of bound protein
- **Streamlined protocol:**
  - Proteolytic release coupled with protease & tag removal
  - One physiological buffer and no need for buffer exchange

Protein Purification from Mammalian Cells

Efficient, Sensitive & Gentle

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Mammalian cells: high quality proteins

- Native environment
- Proper folding
- Protein processing
- Correct post-translation modifications

Low expression level : Low yields; Low recovery; Impurities

HaloTag®:

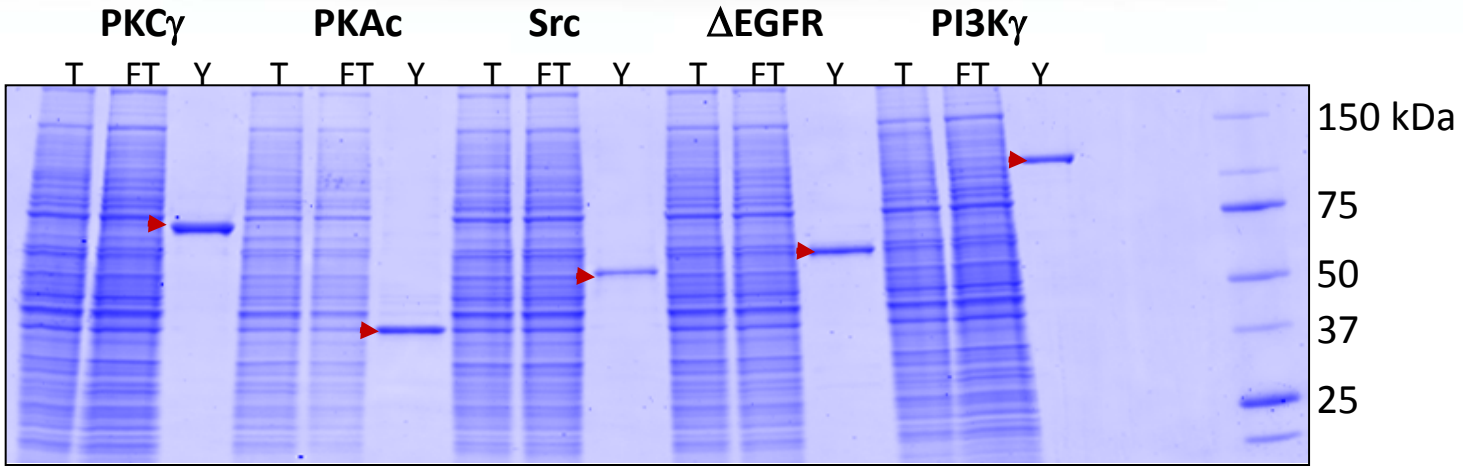
Selective & Covalent capture

- Efficient protein capture regardless of expression levels
- No loss of bound protein during washes

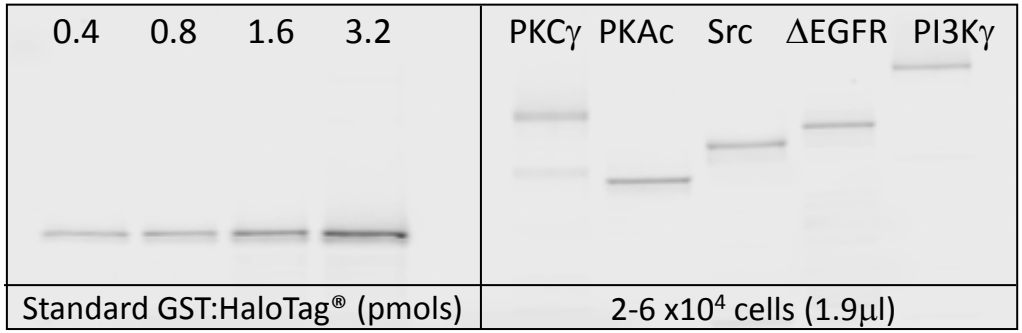
Rapid sensitive detection

- Optimization of expression levels

Purification of Human Kinases from Transient Transfected HEK293T Cells



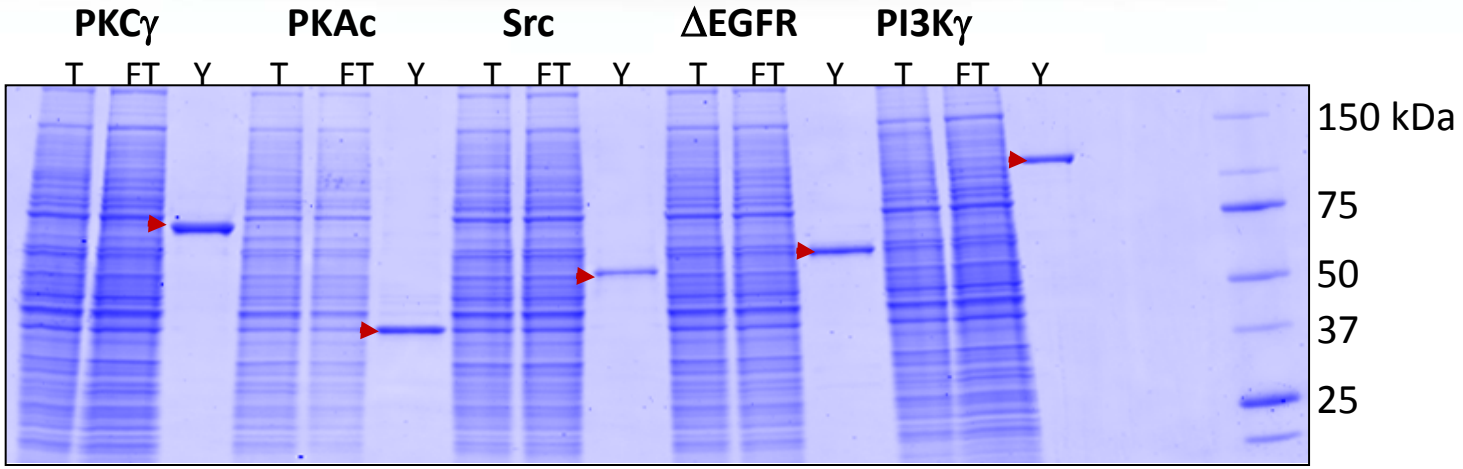
T: starting material **FT:** unbound **Y:** yield



Protein standard and fusions were labeled with HaloTag® TMRDirect ligand

Ohana, R.F., *et al. Prot. Exp. and Purif. (2011)* ***76,*** 154-64

Purification of Human Kinases from Transient Transfected HEK293T Cells



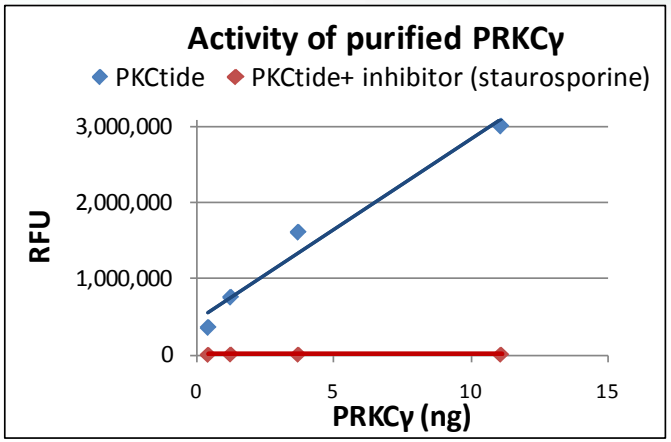
T: starting material **FT:** unbound **Y:** yield

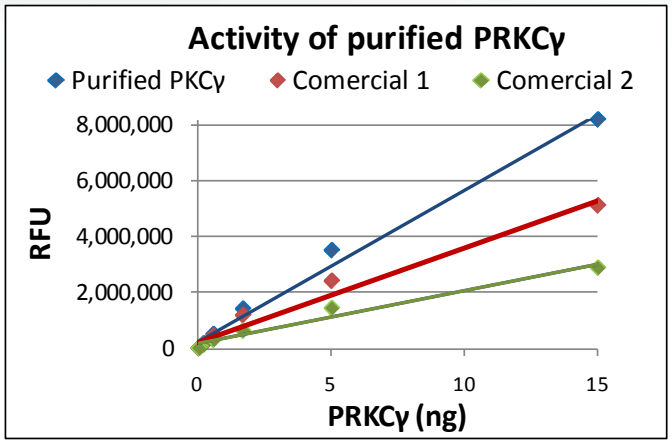
Kinases	PKCγ	PKAc	SRC	ΔEGFR	PI3Kγ
Estimated POI expression (μg)	289	159	181	177	247
Yield (μg)	244	140	137	167	221
% Recovery	**84%**	**88%**	**76%**	**94%**	**89%**

Highly efficient protein capture and recovery

Ohana, R.F., *et al. Prot. Exp. and Purif. (2011)* ***76,*** 154-64

Purified Kinases are Highly Active





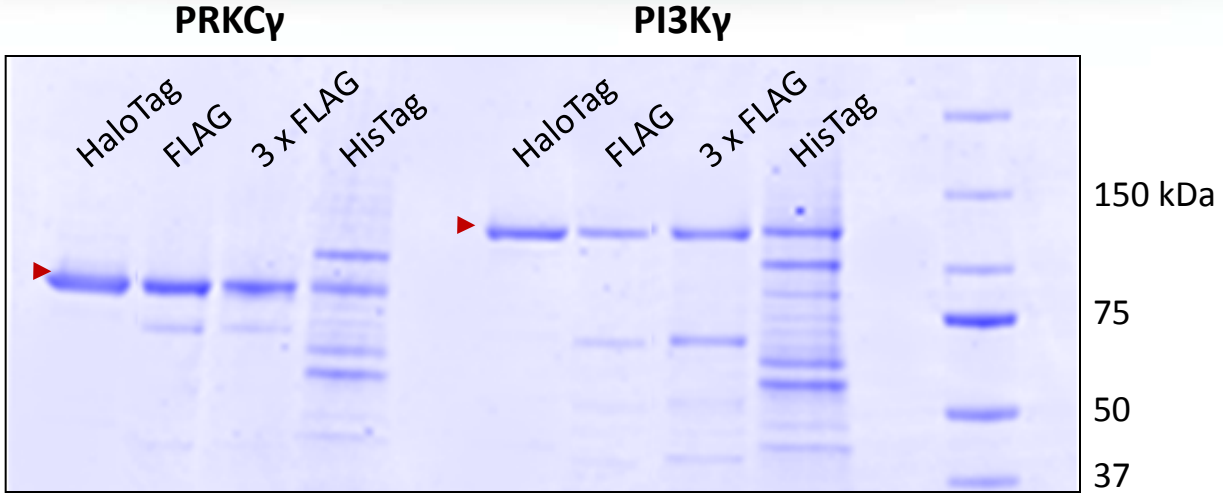
Kinase	Measured Specific activity (nmol/min/mg)	Reported Specific activity (nmol/min/mg)
PRKCγ	16,551	2,260
PKAc	9,670	8,580
Src	1,624	1,032
ΔEGFR	196	101
PI3Kγ	233	39

Measured specific activities are in agreement with reported values

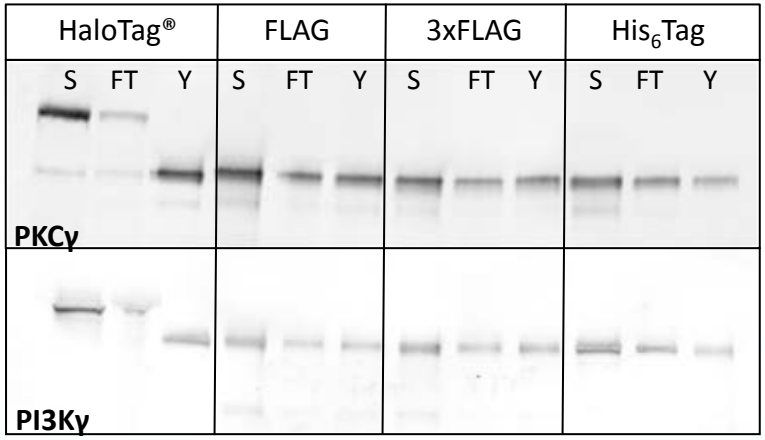
Kinase activity was assayed using ADP-Glo™ assay

Ohana, R.F., *et al. Prot. Exp. and Purif. (2011)* ***76,*** 154-64

Comparative Analysis with Other Affinity Tags



Western analysis



T: starting material **FT:** unbound **Y:** yield

Protein recovery (%)

Tag	PKCγ	PI3Kγ
HaloTag®	86	88
FLAG	60	40
3xFLAG	52	58
His_6Tag	31	33

HaloTag®: Greater protein recovery
Higher yields
Higher purity

Ohana, R.F., *et al. Prot. Exp. and Purif. (2011)* ***76,*** 154-64

Summary: HaloTag®-Based Purification

HaloTag®-based protein purification

- Highly efficient purification regardless of expression levels
- Greater yields, purity and recovery than traditional affinity tags
- Streamlined protocol for proteolytic release coupled with protease & tag removal
- One physiological buffer and no need for buffer exchange

Simple to use fluorescent detection for rapid optimization of expression levels

Studying Intracellular Protein Function

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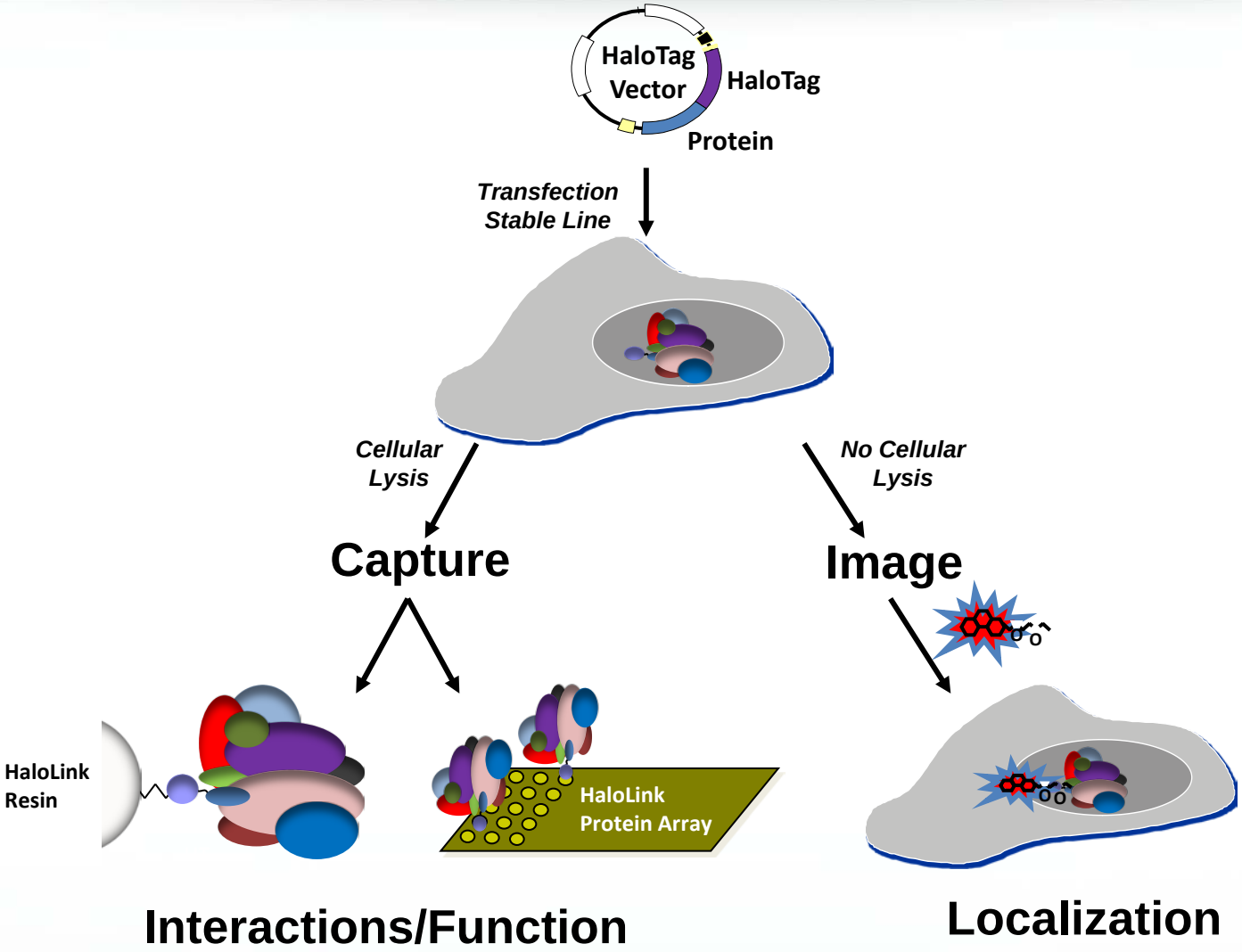
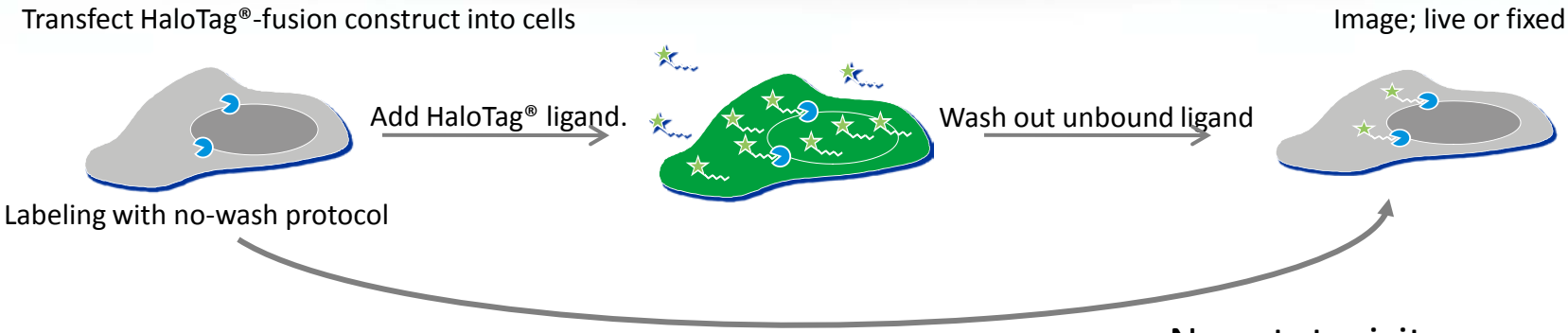
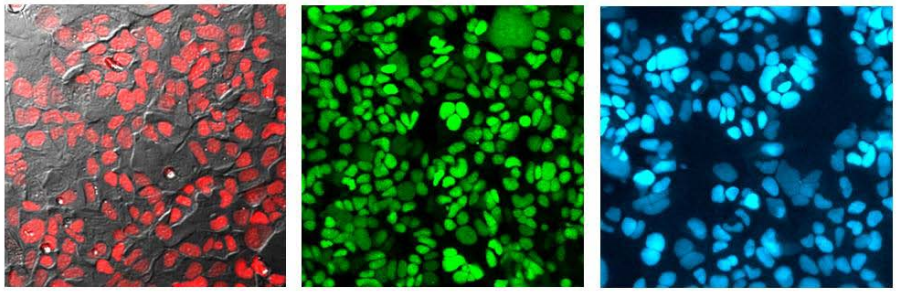


Intracellular Protein Labeling and Imaging



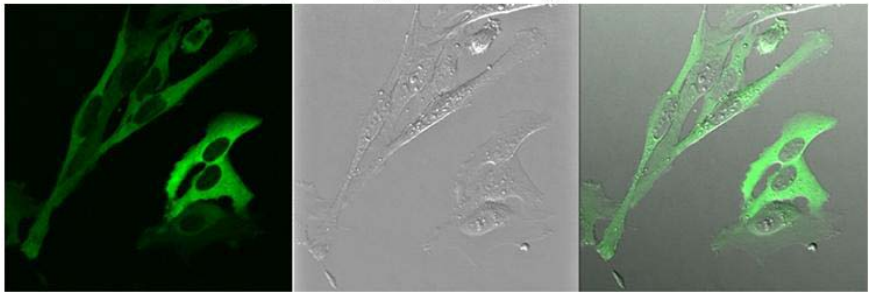
- No cytotoxicity
- Stable labeling
- Multiple colors
- Rapid and easy protocol

HaloTag®-NLS$_3$ fusion protein – HEK293 cells



TMR dAc-FAM Coumarin

U2OS cells stably expressing p65-HaloTag.

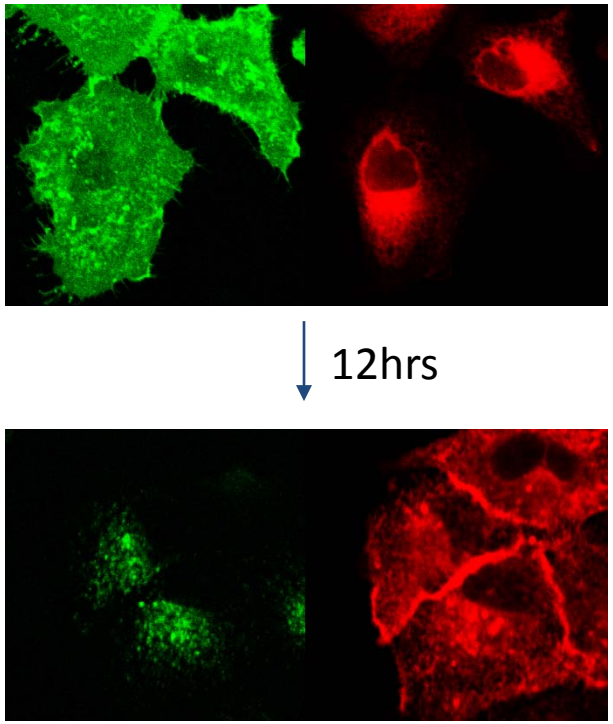


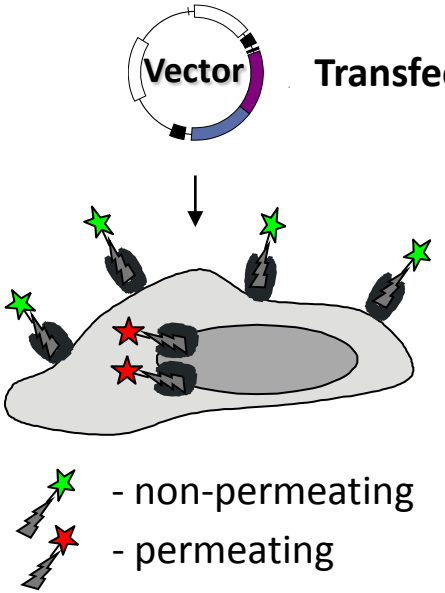
- **All that is done with GFP can be done with HaloTag® and more…**

Analysis of Protein Trafficking

Promega

Label with non-permeant Green → Label with permeant HaloTag® TMR → Wash → Image → Incubate 12 hrs → Image



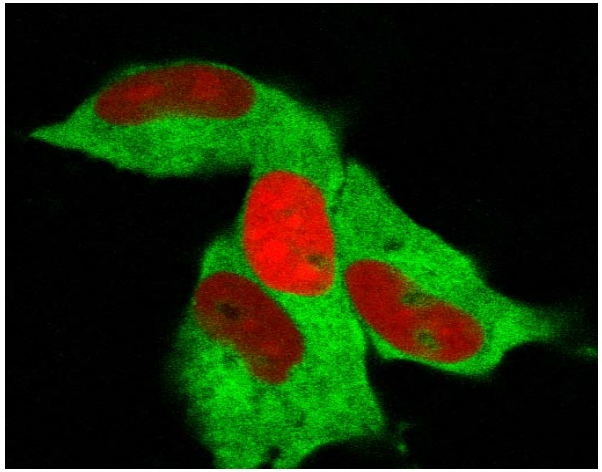


- Spatial control of labeling.
- Follow protein trafficking of distinct protein pools.

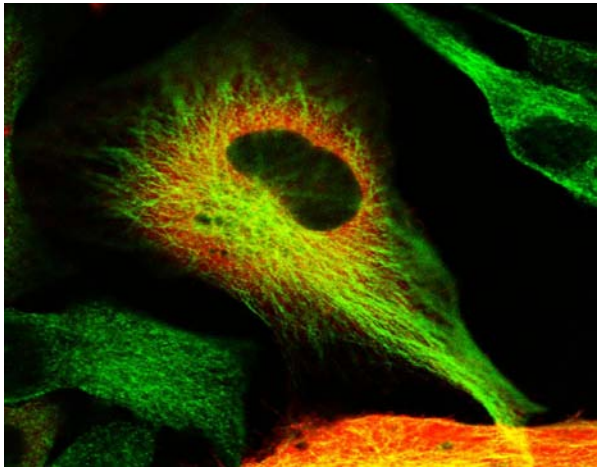
Svendsen, S., et al. *BMC Cell Biol*, 9: 17, 2008.

Multiplexing with Other Labeling Technologies

hMGFP–α-tubulin
HaloTag®–NLS_3-TMR ligand



p65-HaloTag®-TMR Ligand
Alexa 488 Ab



p65-HaloTag labeled withTMR Ligand
fixed, then processed for ICC with anti-β-tubulin Ab
and Alexa-488-conjugated secondary antibody

- HaloTag® is compatible with fluorescent protein fusions
- HaloTag® is compatible with fixing and antibody staining
- Labeling simultaneous with fixation also possible

Real-time Imaging

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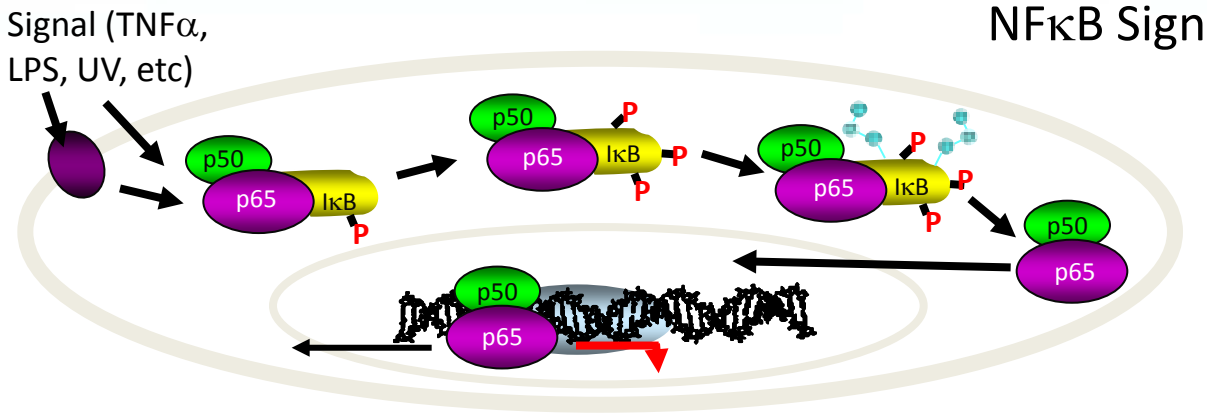
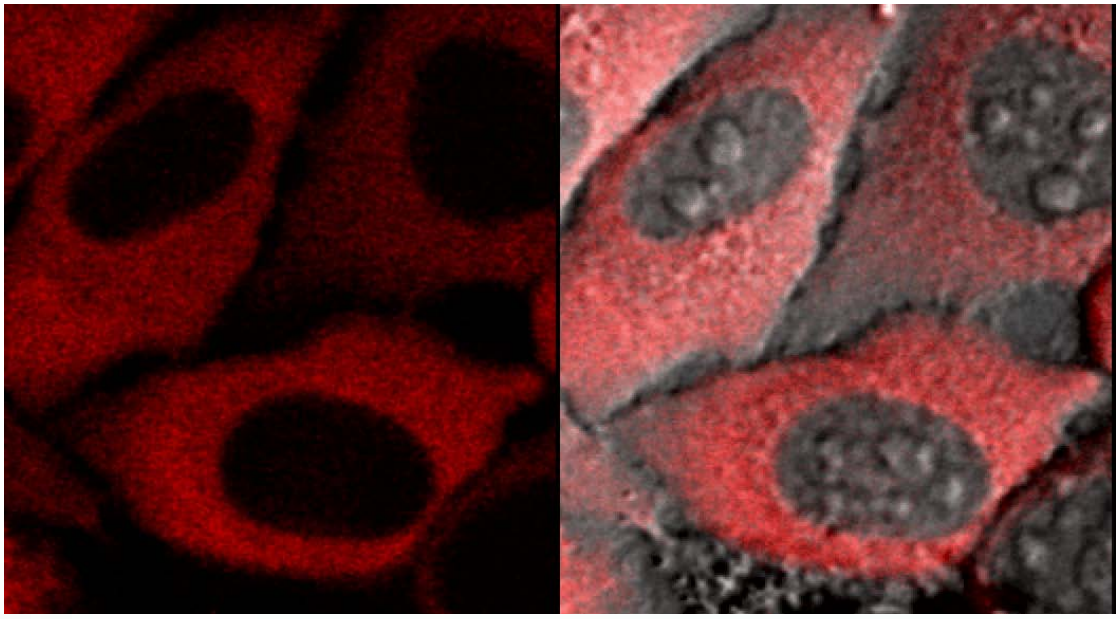
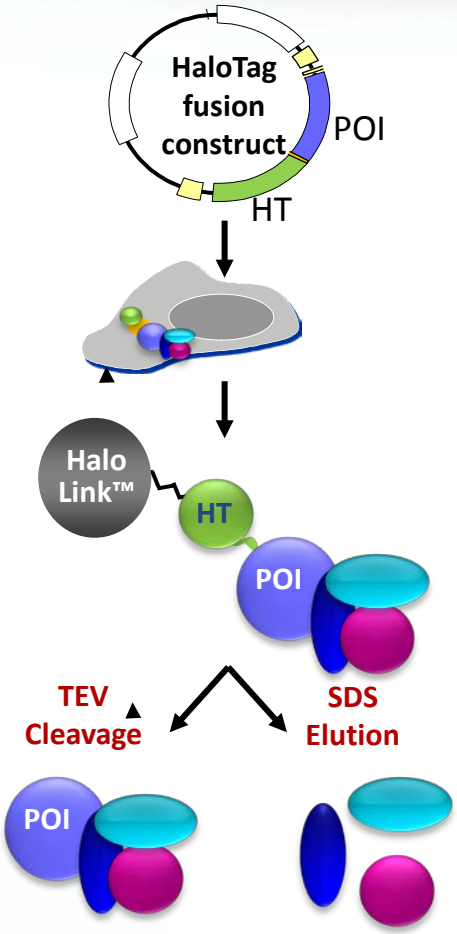
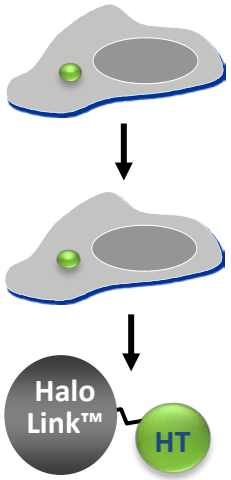


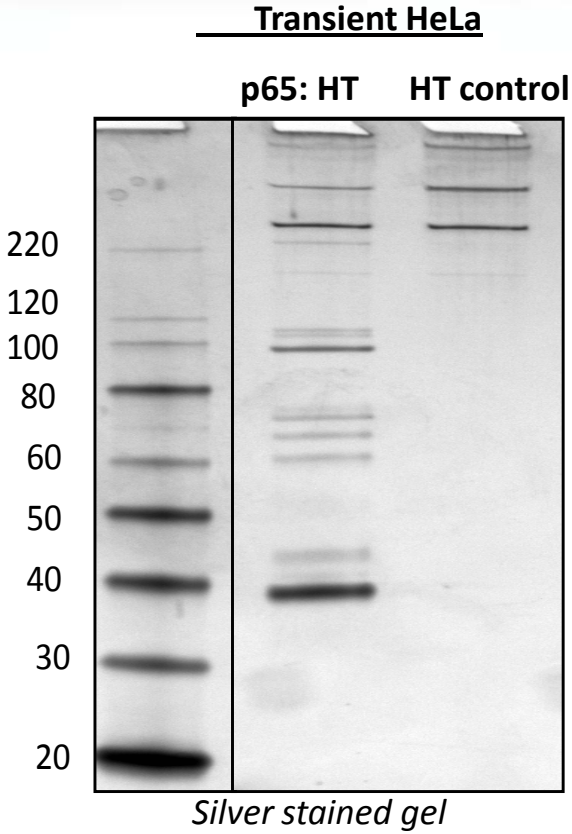


- HeLa cells expressing p65-HaloTag labeled with TMR Ligand
- Treated with TNFα
- Imaged (5min/frame; 120min)

Capture of Protein:Protein Complexes





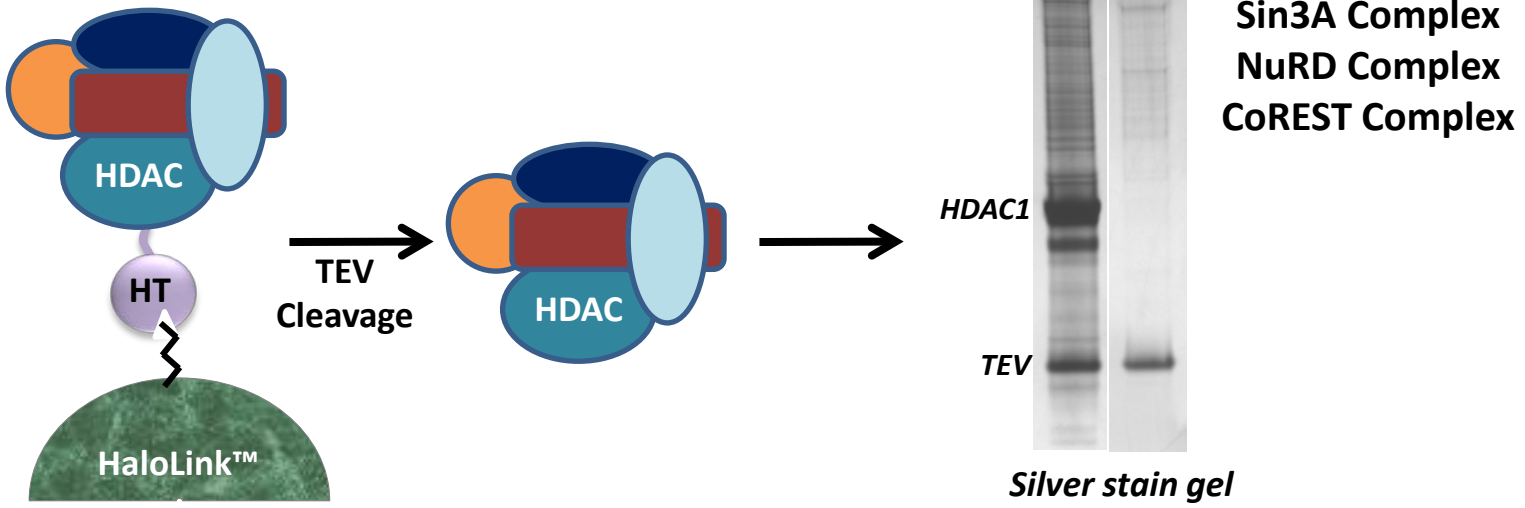


Protein identified by MS analysis:
p105 (p50)
p100
Rel A (p65)
p50
p52
Rel B
C-Rel
IκBα, IκBβ, IκBε

***Same results from TEV cleavage and in-solution digestion**

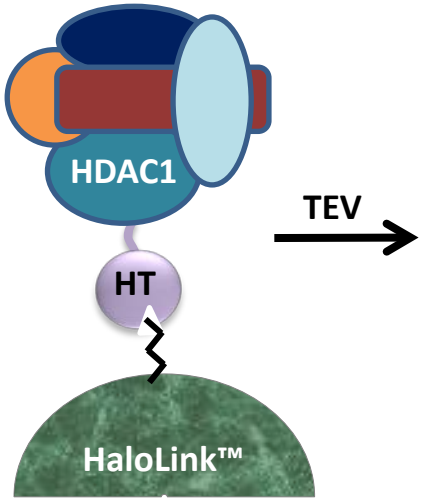
- **p65-HaloTag specifically pull-down expected protein partners of the NFkB pathway**

HDAC1 Complex Purification



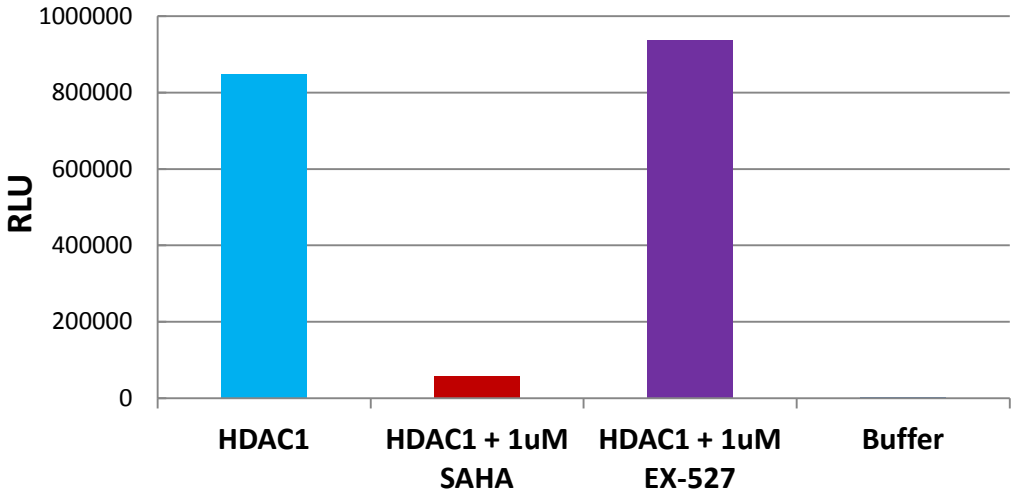
- Expected HDAC complex capture as determined by Mass Spec
- TEV cleavage allows for HDAC complexes to be released in tact.
- Compatible with downstream functional analysis

Isolated HDAC1 Complexes Show Specific Activity



TEV

HDAC-Glo™ +/- HDAC (SAHA) and SIRT (EX-527) inhibitors



- **Enrichment of specific HDAC activity from HDAC complex purification**
- **Able to screen effects of inhibitors on purified physiological complexes**
- **Overall technology extended to other epigenetic complex**

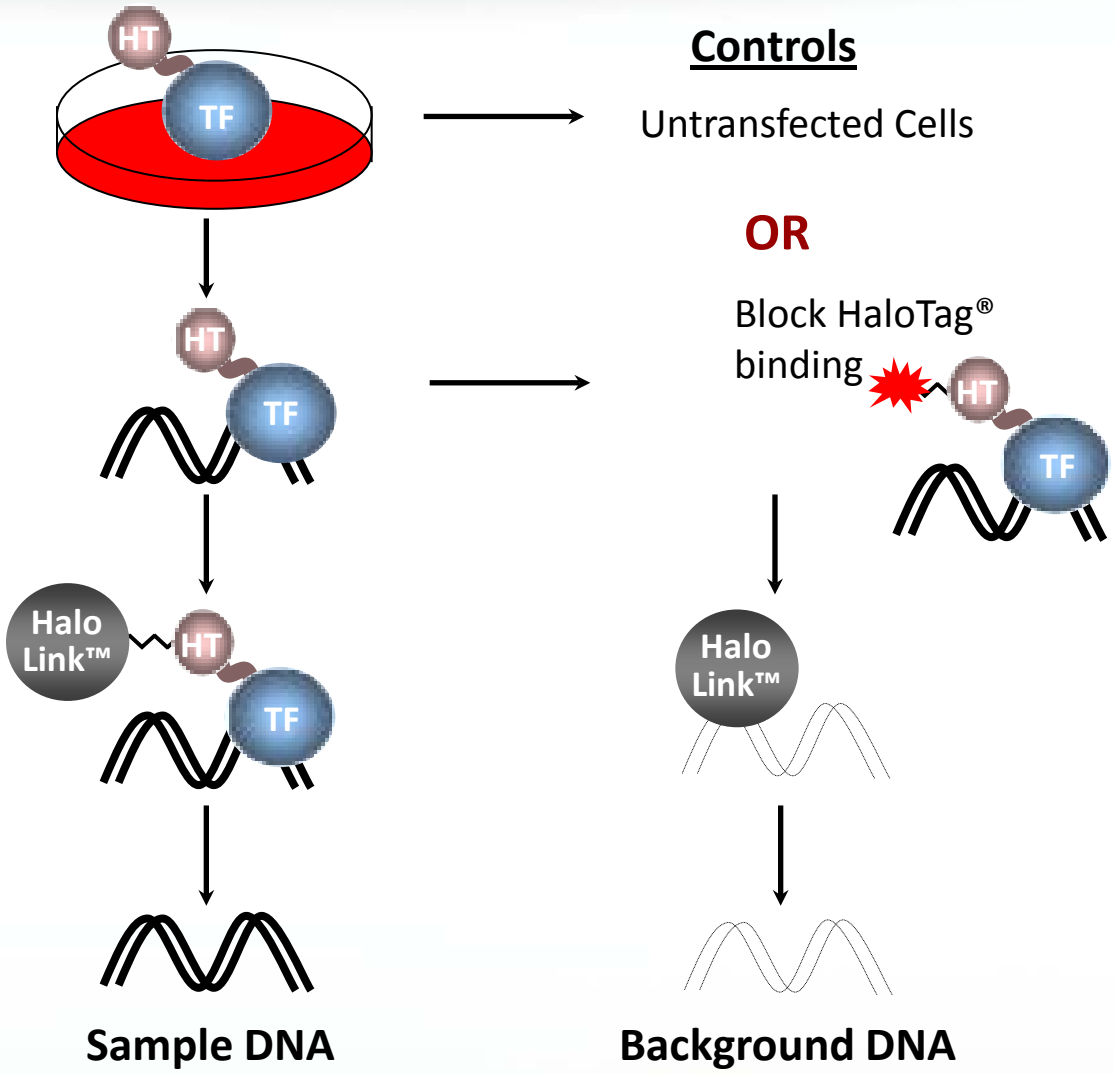
Intracellular Protein:DNA Interactions -HaloCHIP™

1) Expression of HaloTag® (HT) Trxn. factor (TF) fusion protein.

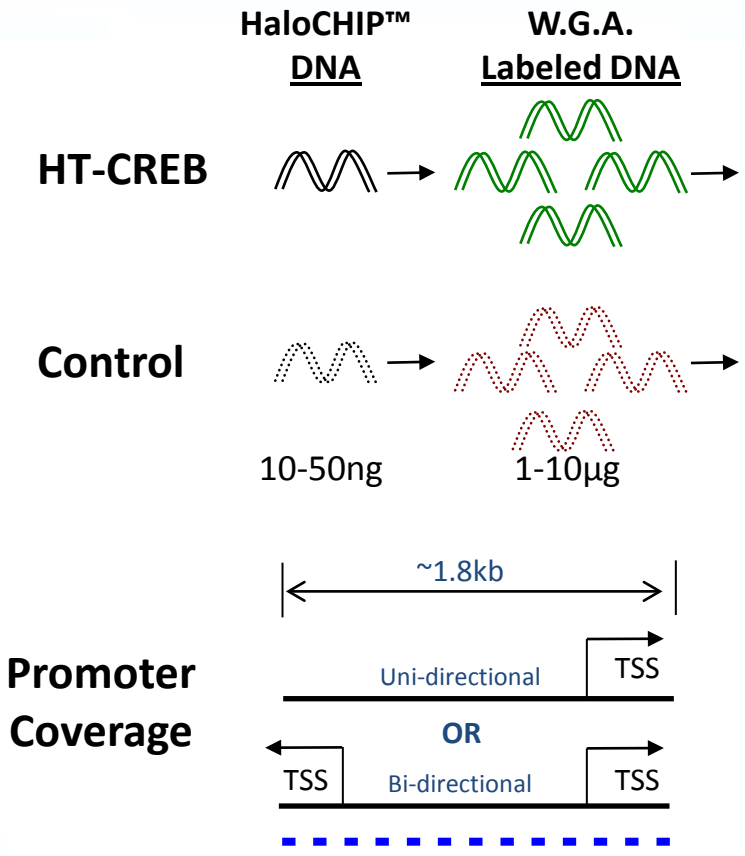
2) Crosslinking, lysis, and sonication.

3) Covalent capture on HaloLink™ resin followed by stringent washing.

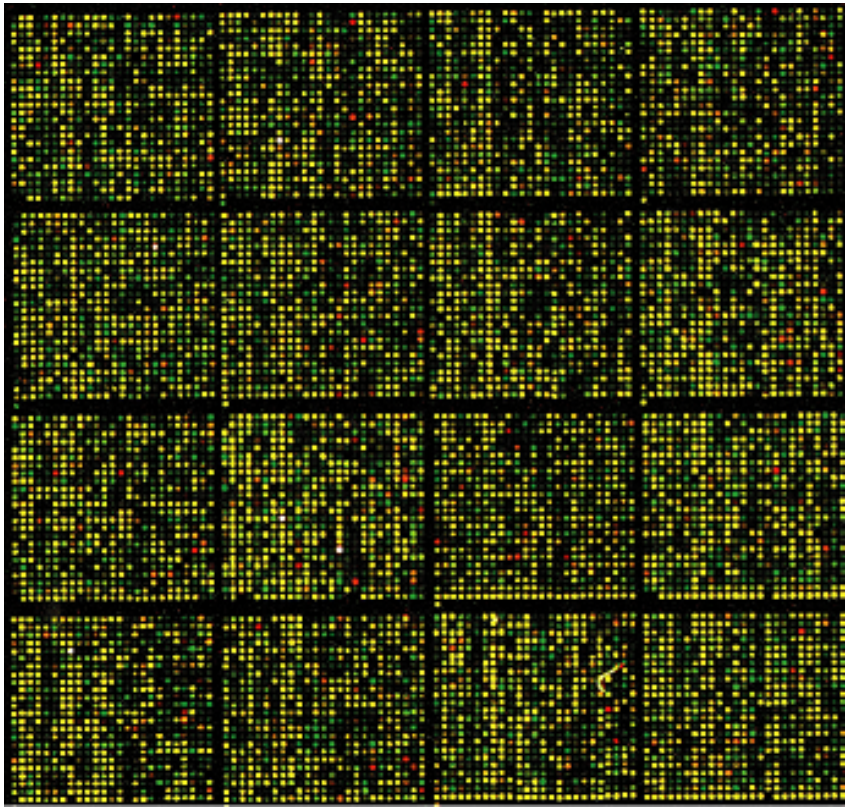
4) Release of DNA by reversal of crosslinks.



CREB HaloCHIP-chip genome wide analysis



CREB HaloCHIP™-chip array



27,661 promoters - 385,000 probes

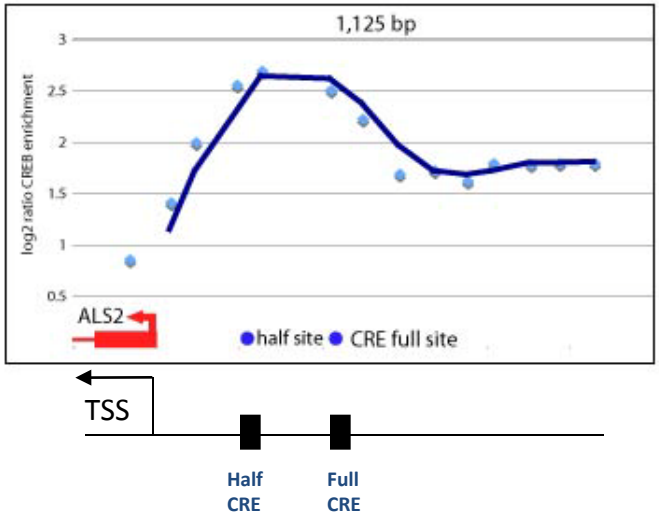
Hartzell, D.D., *et al. BMC Genomics*, **10**: 497 (2009)

Gene Ontology (GO) and Promoter Analysis

CREB HaloCHIP™-ChIP Top 1% Promoters

Cellular Functions	# of Promoters	p-value
Histone Assembly	12/65	1.26E-06
Chromatin architecture	20/261	7.63E-07
Ribonucleic Complexes	26/392	7.06E-07
RNA processing	26/395	8.01E-07
DNA metabolism	38/638	2.93E-08
Nucleic acid binding	110/2764	2.19E-09

Promoter Binding Profile



- **List of promoters all involved in processes CREB is known to regulate.**
- **Binding profile shows peaks binding above CRE consensus sites**

Hartzell, D.D., *et al. BMC Genomics*, **10**: 497 (2009)

HaloCHIP™ and ChIP Binding Patterns

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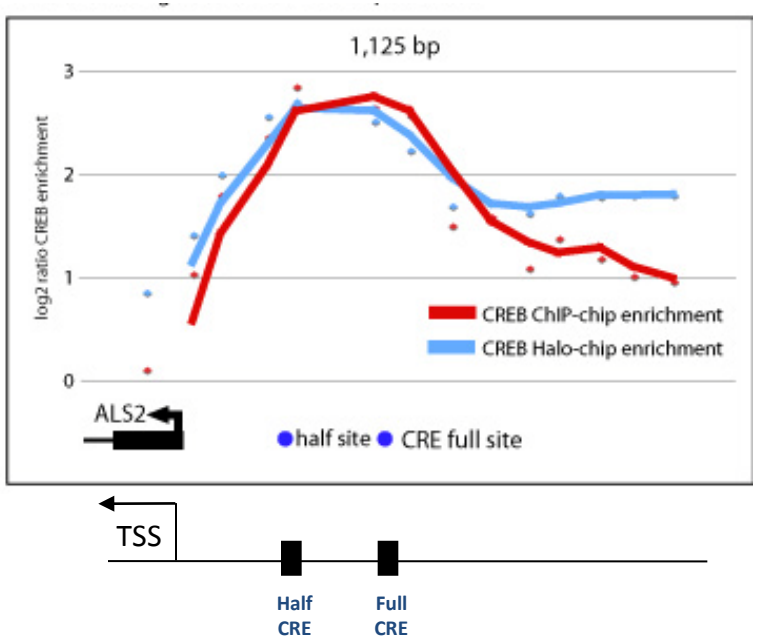
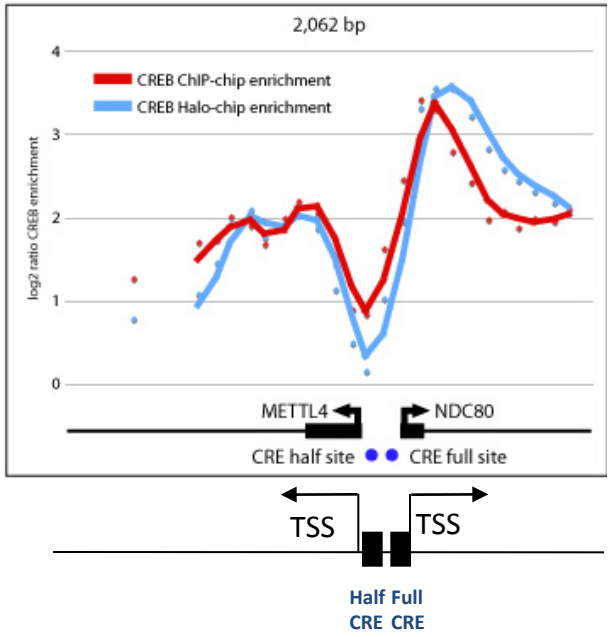
Uni-directional Promoter



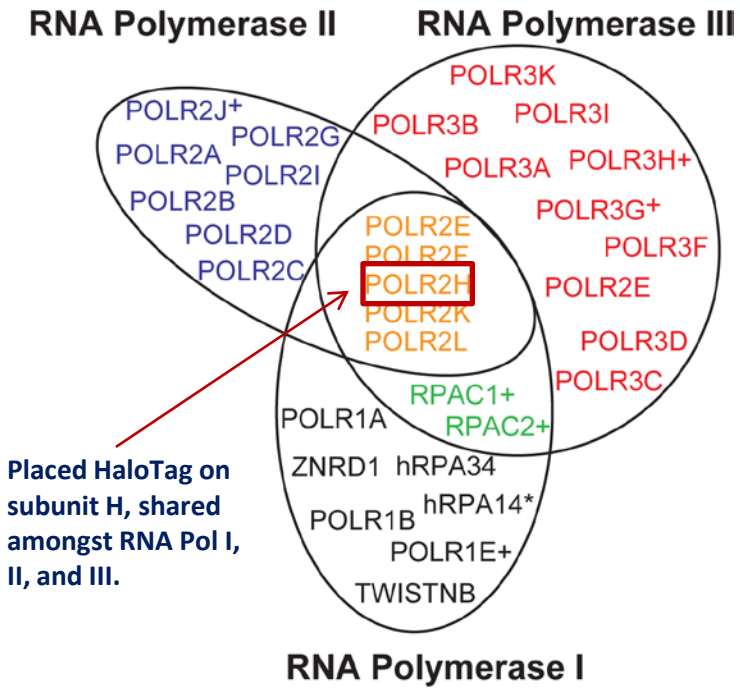
Bi-directional Promoter



- **Overlapping genomic binding patterns between endogenous CREB and Halo-CREB**
- **High percentage of bi-directional promoters showing downstream CREB binding**

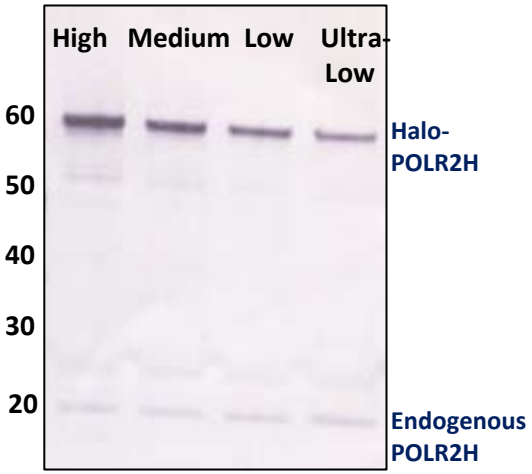
Hartzell, D.D., *et al. BMC Genomics*, **10**: 497 (2009)

Expression Studies and Isolation of Eukaryotic RNA Polymerases



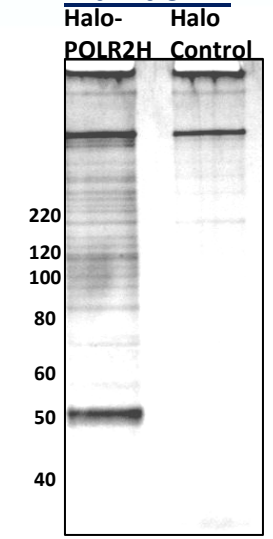
Expression levels of Halo-POLR2H

CMV promoter deletion series



Western – anti-POLR2H

Pull-down



- **A CMV deletion series yielded HT-POLR2H expression over a 50-fold range.**
- **Pull-down performed for each in triplicate and analyzed by MudPIT mass spectrometry.**

Daniels, D.L., *et al. J Proteome Res*, (2012)

Qualitative Data from Triplicate Experiments – RNAP Subunits

	HT Control	HT High	HT Medium	HT Low	HT Ultra
POLR1A		XXX	XXX	XXX	XXX
POLR1B		XXX	XXX	XX	XXX
RPAC1		XXX	XXX	XX	XXX
POLR1D		XXX	XXX	XX	XX
POLR1E		X	XX		X
hRPA34		X	XXX	X	XXX
TWISTNB		X	X	X	
ZNRD1		XX	XX	X	X
POLR2A		XXX	XXX	XXX	XXX
POLR2B		XXX	XXX	XX	XXX
POLR2C		XXX	XXX	XX	XXX
POLR2D		XXX	XXX	XX	XXX
POLR2E		XXX	XXX	XX	XXX
POLR2F		XX			
POLR2G		XXX	XXX	X	XX
POLR2H		XXX	XXX	XXX	XXX
POLR2I		XXX	XX	X	XX
POLR2J		XXX	XXX	XX	XX
POLR2K		X	X		X
POLR2L		XXX	XXX	X	XX
POLR3A		XXX	XXX	XX	XXX
POLR3B		XXX	XXX	X	XXX
POLR3C		XXX	XXX		X
POLR3D		XXX	XXX	XX	XX
POLR3E		XXX	XXX	X	XX
POLR3F		XXX	XXX	X	XXX
POLR3G		XXX	XXX	X	X
POLR3G-LIKE		XX	XX		X
POLR3H		X	XXX		
POLR3I		XX	XXX		X
POLR3K		XXX	XXX	X	XXX
	0 of 31	31 of 31	30 of 31	24 of 31	28 of 31

Number of hits out of 3 replicates

	0 of 3
X	1 of 3
XX	2 of 3
XXX	3 of 3

- **Excellent subunit recovery across CMV deletion series**

Overall recovery out of all possible subunits.

Comparison with FLAG-POLR2H

Promega

Acronym	HT-POLR2H	HT Control	FLAG-POLR2H	FLAG Control
POLR1A	XXX		XX	
POLR1B	XXX		X	
RPAC1	XXX		XXX	XX
POLR1D	XXX			
POLR1E	X			
hRPA34	X		XX	
TWISTNB	X			
ZNRD1	XX			
POLR2A	XXX		XXX	X
POLR2B	XXX		XXX	
POLR2C	XXX		XX	
POLR2D	XXX		X	
POLR2E	XXX		X	
POLR2F	XX			
POLR2G	XXX			
POLR2H	XXX		XXX	
POLR2I	XXX			
POLR2J	XXX			
POLR2K	X			
POLR2L	XXX			
POLR3A	XXX		X	
POLR3B	XXX			
POLR3C	XXX		X	
POLR3D	XXX		XX	
POLR3E	XXX			
POLR3F	XXX			
POLR3G	XXX			
POLR3G-LIKE	XX			
POLR3H	X			
POLR3I	XX			
POLR3K	XXX			

Number of times core RNAP subunits identified by MudPIT analysis in triplicate experiments.

	0 of 3
X	1 of 3
XX	2 of 3
XXX	3 of 3

- **Improved recovery with HaloTag**
- **Higher reproducibility**
- **Lower background**

Daniels, D.L., *et al. J Proteome Res*, (2012)

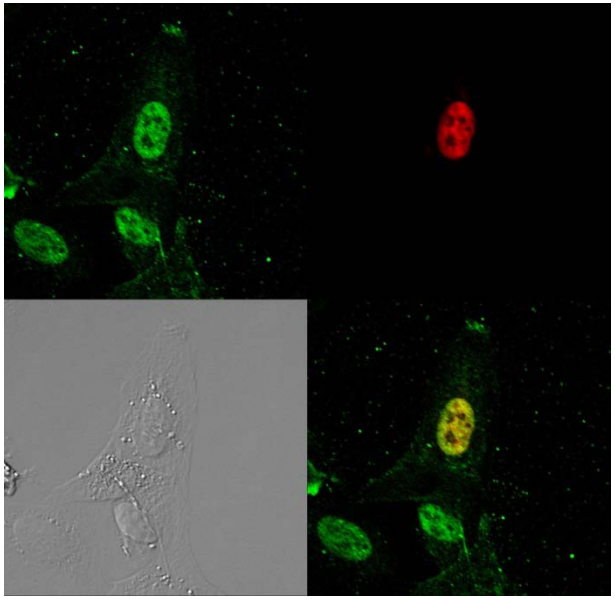
Imaging of HaloTag®-POLR2H

Promega

Co-localization with Endogenous POLR2A

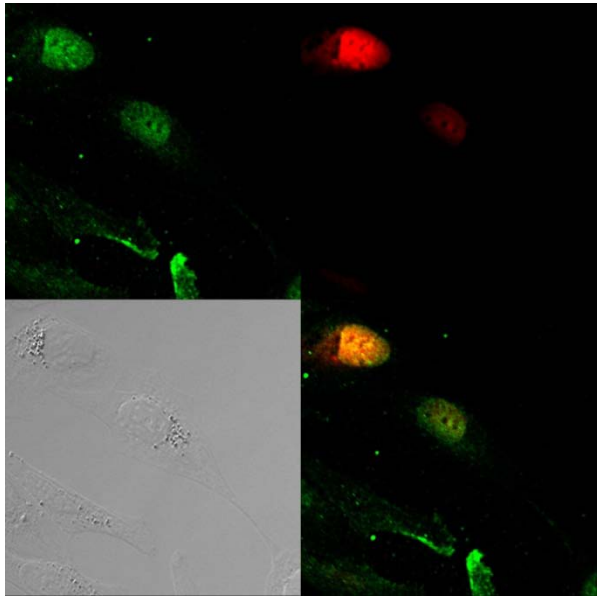
Antibody POLR2A(CTD)

TMR HT-POLR2H



Antibody POLR2A(CTD)

TMR HT-POLR2H



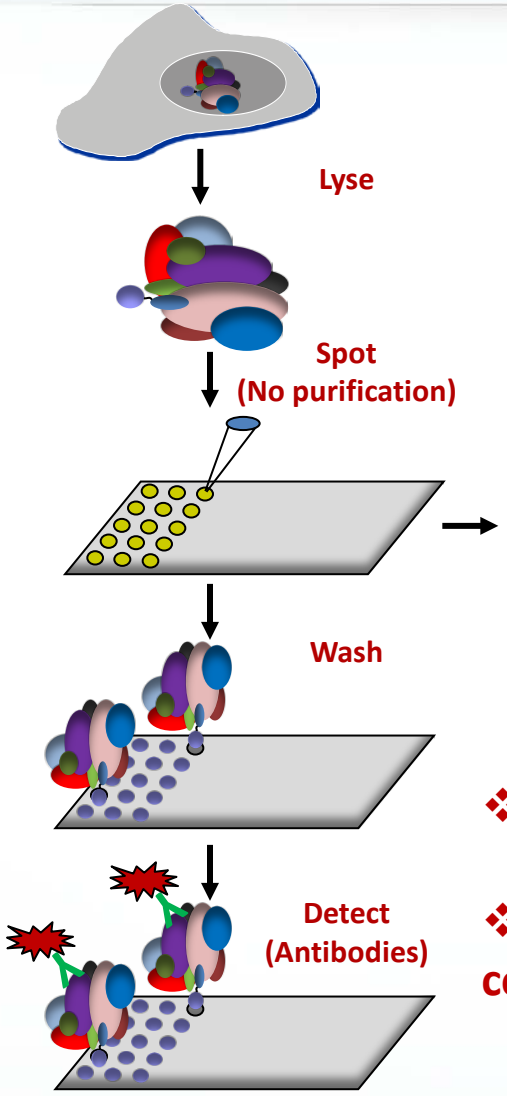
←Overlay

- **HaloTag-POLR2H is properly localized to the nucleus.**
- **Co-localization with antibody specific to the CTD of POLR2A.**

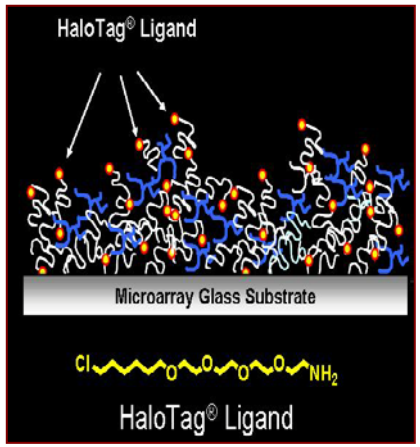
Daniels, D.L., *et al. J Proteome Res*, (2012)

Oriented Capture of HaloTag-POLR2H on HaloLink™ Arrays

Promega



HaloLink™ Slide Surface
(PEG linker with Chloroalkane)



HaloTag-POLR2H
Capture on Slide

Antibodies

POLR2A
(CTD)

HaloTag

Experimental
(Halo-POLR2H)

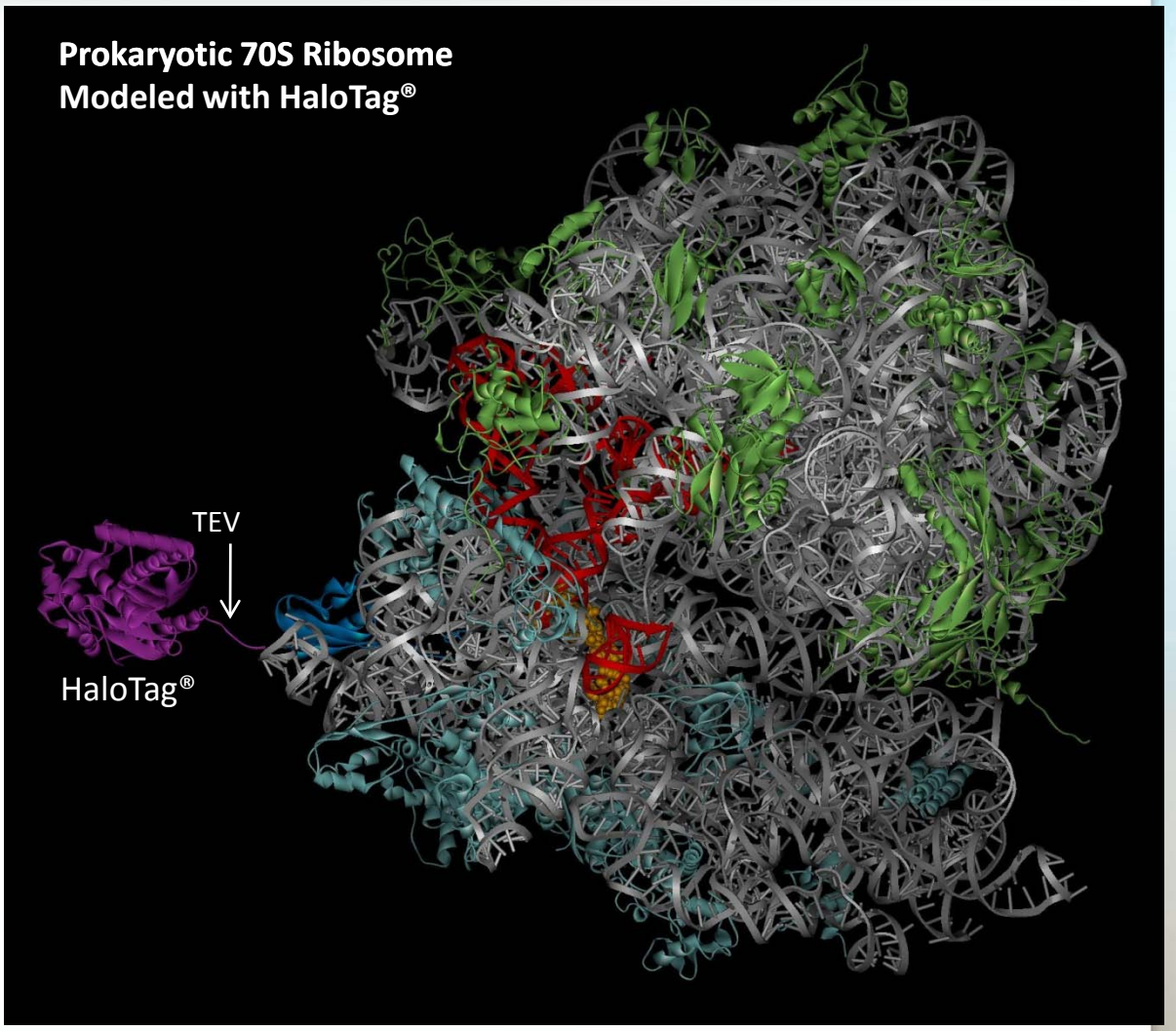
Control
(HaloTag)

❖**HaloTag-POLR2H isolated on slide directly from lysate.**

❖**Detection of binding partner suggests RNA Polymerase complex capture on slides.**

Isolation of the Ribosome

- One of the largest macromolecular machines.
- Highly abundant.
- Many interacting partners, mRNAs.
- Difficult to capture using a single protein fusion tag.
- Placed HaloTag® on 40S subunit protein, Human RPS9.

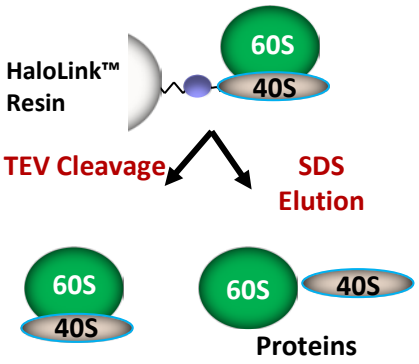


Selmer *et al.* (2006) *Science* **313**, 1935 pdb:2j00 and 2j01 – 70S Ribosome

Capture of Eukaryotic Ribosomes Using S9-HT

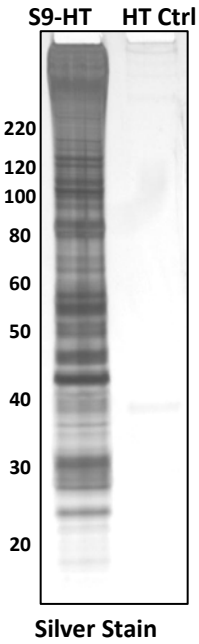
Promega

S9-HT Pull-down



- HaloTag® remains on resin
- Rapid binding -15min.
- No diffusion off resin
- Capture low levels of protein

TEV Cleavage

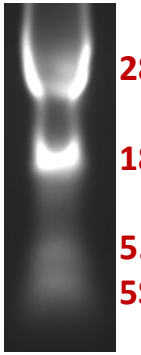


Identified by MS (LC-MS/MS)

- 31 of 33 40S proteins
- 42 of 50 60S proteins
- 2 Poly-A binding proteins
- 1 GNF exchange protein
- 9 Nuclear ribonucleoproteins
- 2 Initiation factors
- 2 Elongation factors
- 2 Splicing factors

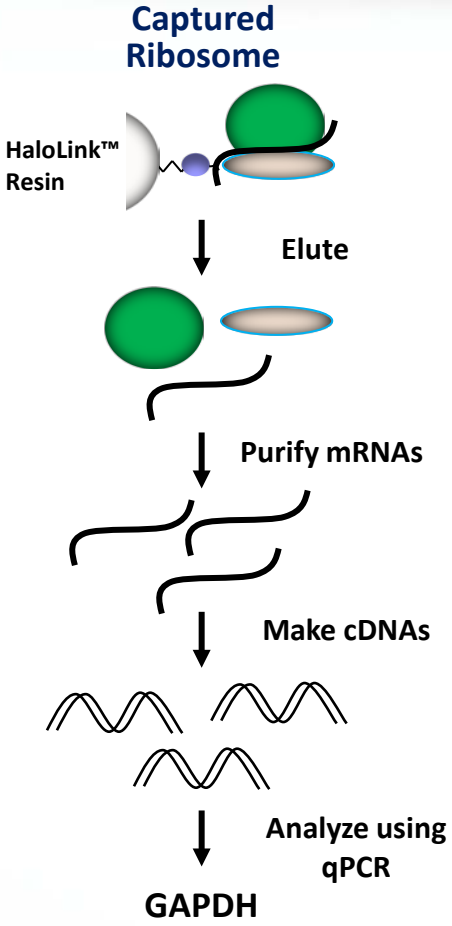
RNA Capture

Purified RNAs

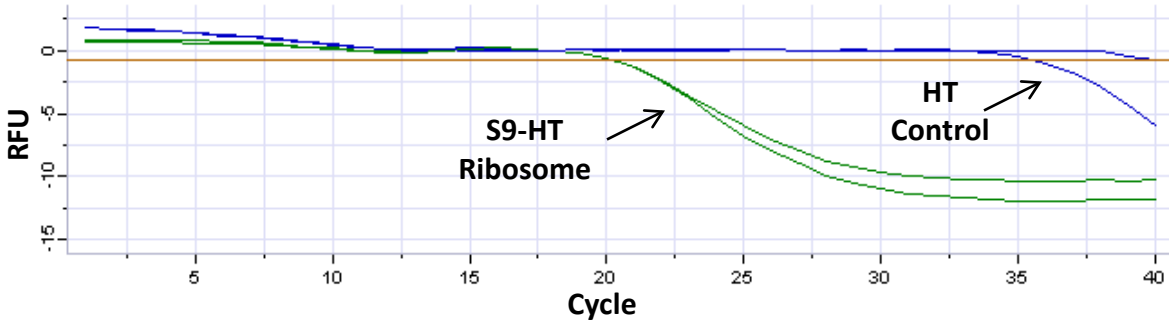


- **Purified the 80S ribosome with single step from HeLa cells.**
- **Efficiently cleave complex from resin with TEV protease.**

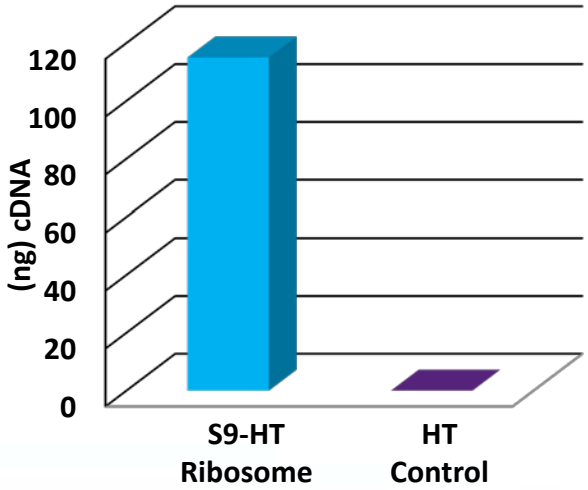
Isolated Ribosomes are Bound to mRNAs



Plexor qPCR GAPDH cRNA Amplification



Total GAPDH cDNA



- GAPDH mRNA bound to isolated ribosomes
- Potential for complete mRNA analysis.

Isolated Ribosomes are Active for Translation

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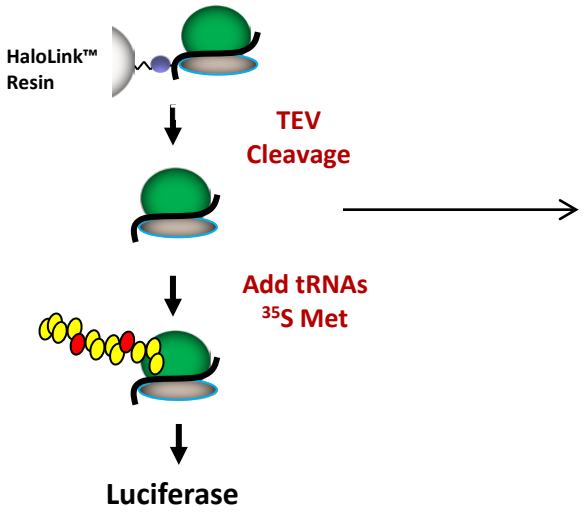
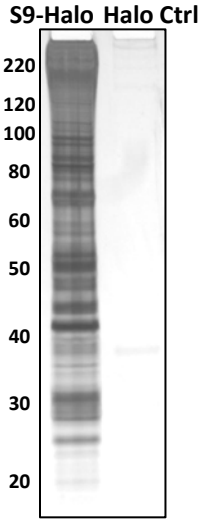
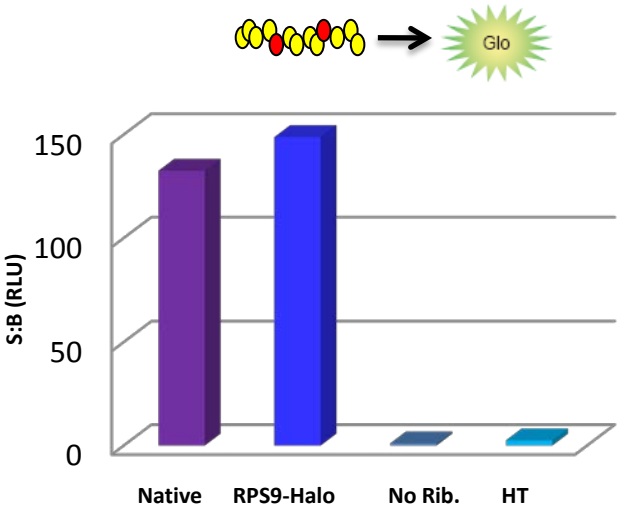
Pulldown of Luc-mRNA RPS9-HT in Luc-HEK293



Ribosome Pulldown



Specific luciferase synthesis

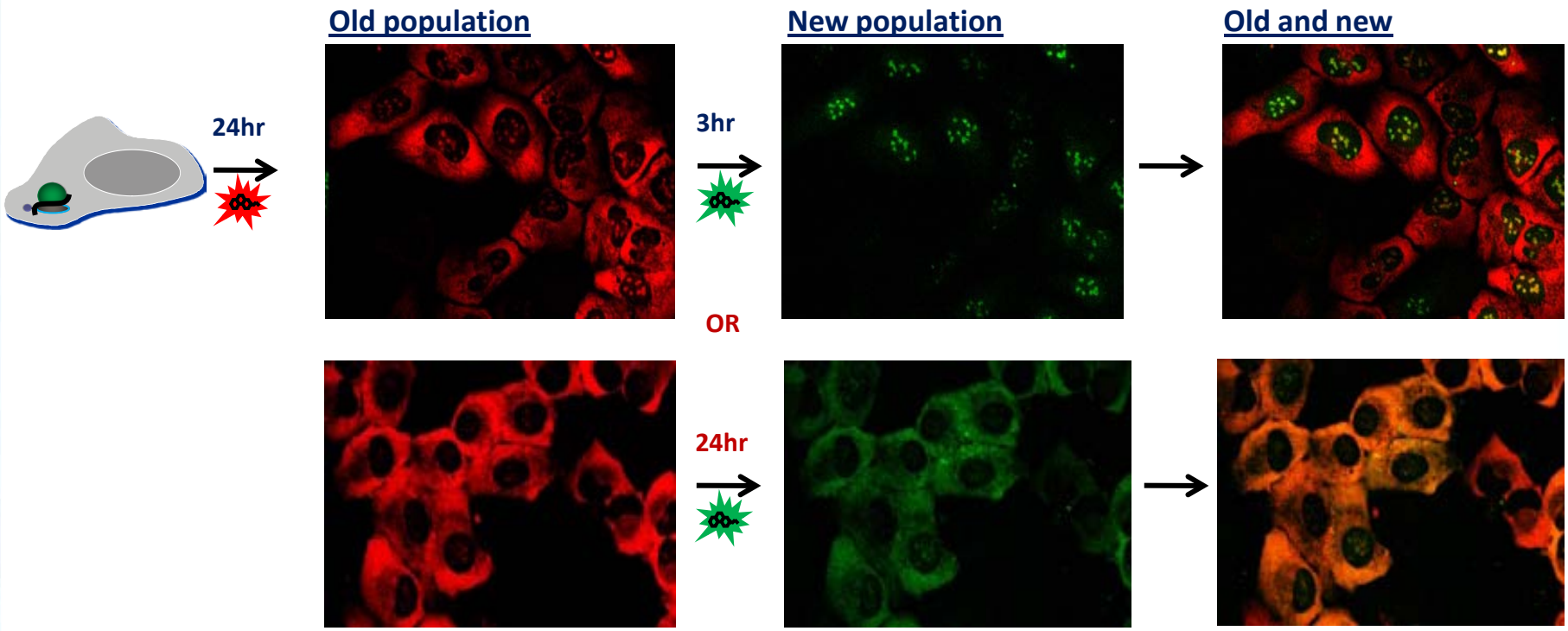


- **Ribosomes which have incorporated RPS9-Halo are active for elongation.**
- **Bound to Luc-mRNA and can translate functional luciferase protein.**

Monitoring Ribosomal Trafficking and Populations

Promega

Serum Starve RPS9-HT | Pulse TMR Label | Serum Recovery | Chase Green Label | Wash | Image Overlay

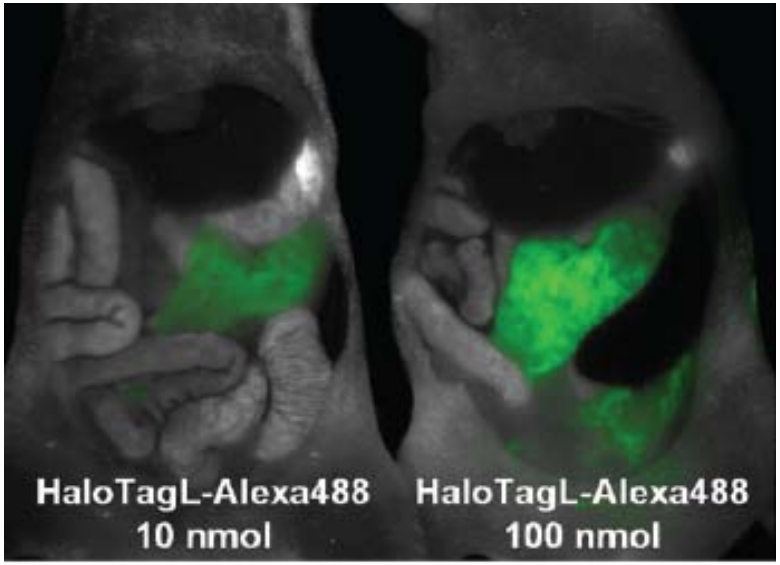


- Newly synthesized ribosomal proteins localized to nucleoli 3hrs post-stress.
- After 24hrs of recovery, ribosomal populations re-localized to cytoplasm.

HaloTag® Platform

- In vivo fluorescent imaging
- Future directions

In Vivo Imaging



Fluorescent *in vivo* imaging using HaloTag® technology allows for development of imaging ligands

- **PET**
- **near IR**

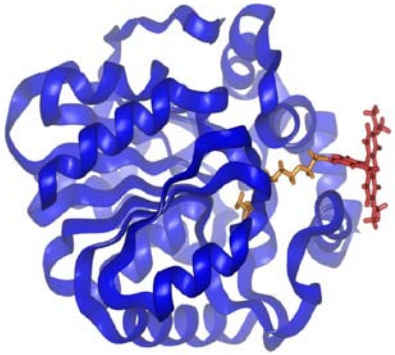
Summary

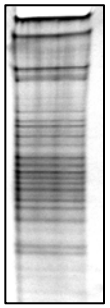
Promega

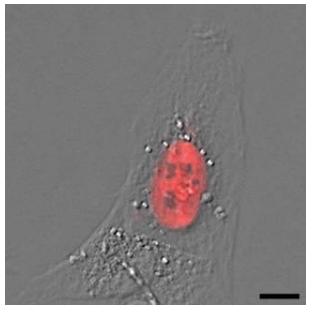
- **Evolved HaloTag® protein for specific, covalent, and rapid binding.**

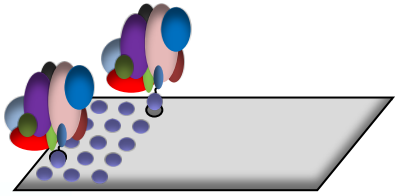
- **HaloTag® technology shows strength and advantages for a variety of mammalian applications:**

  - ***Protein purification***
  - ***Capture on surfaces***
  - ***Protein:DNA interactions***
  - ***Protein complex isolation***
  - ***Cellular and in vivo imaging***









Glass Slides