Overcoming Challenges of Protein Analysis in Mammalian Systems

Danette L. Daniels, Ph.D.
Current Technologies for Protein Analysis

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

How about a system applicable to all approaches that also addresses limitations of current methods?

- Biochemical/Proteomic Analysis
- Affinity tags
- Antibodies
- Cell Based Analysis
- In Vivo Animal Models
HaloTag Platform

Biochemical/Proteomic Analysis
- Protein purification
- Protein arrays
- Protein interactions

Cell Based Analysis
- Protein localization
- Real time imaging
- Protein trafficking
- Protein turnover

In Vivo Animal Models
- 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**

**Hydrolase (DhaA)**

Catalytic process

**HaloTag®**

Facilitated bond formation

**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® protein
- HaloTag® ligand
- 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 functionalities**: a single fusion construct may be attached to a broad range of functional properties
**Generation of a N- & C-Terminal HaloTag® Fusions**

**Flexi vectors for expression of HaloTag fusions in mammalian cells:**

<table>
<thead>
<tr>
<th>Expression</th>
<th>Promoter</th>
<th>N-terminal HaloTag®</th>
<th>C-terminal HaloTag®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal expression</td>
<td>CMV</td>
<td>pFN21</td>
<td>pFC14</td>
</tr>
<tr>
<td>Promoter deletion series to optimize mammalian expression level</td>
<td>CMVd1</td>
<td>pFN22</td>
<td>pFC15</td>
</tr>
<tr>
<td></td>
<td>CMVd2</td>
<td>pFN23</td>
<td>pFC16</td>
</tr>
<tr>
<td></td>
<td>CMVd3</td>
<td>PFN24</td>
<td>pFC17</td>
</tr>
</tbody>
</table>

**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**

**Kazusa DNA Research Institute**

Human ORFs N-terminal fusion constructs in Flexi® vector pFN21A for expression in mammalian cells


<table>
<thead>
<tr>
<th>Features</th>
<th>Flexi-HaloTag Collection PID beginning with FHC as of Nov, 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of Collection</td>
<td>7,100 clones</td>
</tr>
<tr>
<td>Fusion Tag</td>
<td>HaloTag®</td>
</tr>
</tbody>
</table>

**Validated Clones**

- Sequence Validated: Yes (100% clones)
- Insert Validated: Yes (99.7% clones)
- Expression Validated: Yes (99.3% clones)
- Localization Validated: Yes (80.1% clones)

<table>
<thead>
<tr>
<th>Format</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical Delivery</td>
<td>2-4 weeks</td>
</tr>
<tr>
<td>Price (USD)</td>
<td>$500.00 per clone</td>
</tr>
</tbody>
</table>

**GeneCopoeia**

Human and mouse ORFs N- /C- terminal fusion constructs available in OmicsLink™ vectors for expression in mammalian cells

**HaloTag®-based Protein Purification Scheme**

- **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

**HaloLink**

- Protein of Interest
- HT

**Proteolytic release**

- POI proteolytically released via HaloTEV
- HaloTEV & HT remain permanently attached to HaloLink

**Recovery**

- Protein free of tag
**Protein Purification from Mammalian Cells**  
**Efficient, Sensitive & Gentle**

**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

PKC\(\gamma\)  PKAc  Src  \(\Delta\)EGFR  PI3K\(\gamma\)
T  FT  Y  T  FT  Y  T  FT  Y  T  FT  Y

T: starting material  FT: unbound  Y: yield

<table>
<thead>
<tr>
<th>Standard GST:HaloTag(^\circledR) (pmols)</th>
<th>2-6 x10^4 cells (1.9(\mu)l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC(\gamma)  PKAc  Src  (\Delta)EGFR  PI3K(\gamma)</td>
<td>PKC(\gamma)  PKAc  Src  (\Delta)EGFR  PI3K(\gamma)</td>
</tr>
<tr>
<td>0.4  0.8  1.6  3.2</td>
<td></td>
</tr>
</tbody>
</table>

Protein standard and fusions were labeled with HaloTag\(^\circledR\) TMRDirect ligand

Purification of Human Kinases from Transient Transfected HEK293T Cells

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

Purified Kinases are Highly Active

Kinase activity was assayed using ADP-Glo™ assay. Measured specific activities are in agreement with reported values.

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

Measured specific activities are in agreement with reported values

Comparative Analysis with Other Affinity Tags

Western analysis

<table>
<thead>
<tr>
<th>HaloTag®</th>
<th>FLAG</th>
<th>3xFLAG</th>
<th>His6Tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCγ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3Kγ</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tag</td>
</tr>
<tr>
<td>HaloTag®</td>
</tr>
<tr>
<td>FLAG</td>
</tr>
<tr>
<td>3xFLAG</td>
</tr>
<tr>
<td>His6Tag</td>
</tr>
</tbody>
</table>

HaloTag®: Greater protein recovery
Higher yields
Higher purity

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

1. **Transfection Stable Line**
2. **Capture**
   - **Cellular Lysis**
   - **No Cellular Lysis**
3. **Image**
   - **Localization**
4. **Interactions/Function**

- **HaloTag Vector**
- **HaloTag Protein**
- **HaloLink Resin**
- **HaloLink Protein Array**
**Intracellular Protein Labeling and Imaging**

Transfect HaloTag®-fusion construct into cells

Add HaloTag® ligand.

Wash out unbound ligand

Image; live or fixed cells

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

**Labeling with no-wash protocol**

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

---

**HaloTag®-NLS₂ fusion protein – HEK293 cells**

- TMR
- dAc-FAM
- Coumarin

**U2OS cells stably expressing p65-HaloTag.**
Analysis of Protein Trafficking

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.

Multiplexing with Other Labeling Technologies

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

hMGFP–α-tubulin
HaloTag®–NLS₃-TMR ligand

p65-HaloTag®-TMR Ligand
Alexa 488 Ab

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

- Treated with TNFα
- Imaged (5min/frame; 120min)
Capture of Protein:Protein Complexes

**HaloTag fusion construct**

**TEV Cleavage**

**SDS Elution**

**Silver stained gel**

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 NFκB 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

**Identified by MS**
- Sin3A Complex
- NuRD Complex
- CoREST Complex

**Silver stain gel**
- Halo-HDAC1
- Halo-Control
- HDAC1
- TEV
Isolated HDAC1 Complexes Show Specific Activity

- 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 complexes
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.

Controls

- Untransfected Cells
- Block HaloTag® binding

Sample DNA

Background DNA
CREB HaloCHIP-chip genome wide analysis

HaloCHIP™ DNA → W.G.A. Labeled DNA

HT-CREB

Control

10-50ng → 1-10µg

~1.8kb

Promoter Coverage

Uni-directional → TSS

OR

TSS → Bi-directional → TSS

CREB HaloCHIP™-chip array

27,661 promoters - 385,000 probes

### Gene Ontology (GO) and Promoter Analysis

**CREB HaloCHIP™-ChIP Top 1% Promoters**

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

**Promoter Binding Profile**

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

**HaloCHIP™ and ChIP Binding Patterns**

**Uni-directional Promoter**
- 1,125 bp
- Overlapping genomic binding patterns between endogenous CREB and Halo-CREB
- High percentage of bi-directional promoters showing downstream CREB binding

**Bi-directional Promoter**
- 2,062 bp

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.

### Qualitative Data from Triplicate Experiments – RNAP Subunits

<table>
<thead>
<tr>
<th>Subunit</th>
<th>HT Control</th>
<th>HT High</th>
<th>HT Medium</th>
<th>HT Low</th>
<th>HT Ultra</th>
</tr>
</thead>
<tbody>
<tr>
<td>POLR1A</td>
<td>XXX</td>
<td>XXX</td>
<td>XXX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR1B</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>RPAC1</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR1D</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR1E</td>
<td>X</td>
<td>XX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hRPA34</td>
<td>X</td>
<td>XXX</td>
<td>X</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>TWISTNB</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZNDR1</td>
<td>XX</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR2A</td>
<td>XXX</td>
<td>XXX</td>
<td>XXX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR2B</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR2C</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR2D</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR2E</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR2F</td>
<td>XX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR2G</td>
<td>XXX</td>
<td>XXX</td>
<td>X</td>
<td>XX</td>
<td></td>
</tr>
<tr>
<td>POLR2H</td>
<td>XXX</td>
<td>XXX</td>
<td>XXX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR2I</td>
<td>XXX</td>
<td>XX</td>
<td>X</td>
<td>XX</td>
<td></td>
</tr>
<tr>
<td>POLR2J</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>POLR2K</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR2L</td>
<td>XXX</td>
<td>XXX</td>
<td>X</td>
<td>XX</td>
<td></td>
</tr>
<tr>
<td>POLR3A</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR3B</td>
<td>XXX</td>
<td>XXX</td>
<td>X</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR3C</td>
<td>XXX</td>
<td>XXX</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>POLR3D</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3E</td>
<td>XXX</td>
<td>XXX</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3F</td>
<td>XXX</td>
<td>XXX</td>
<td>X</td>
<td>XXX</td>
<td></td>
</tr>
<tr>
<td>POLR3G</td>
<td>XXX</td>
<td>XXX</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3G-LIKE</td>
<td>XX</td>
<td>XX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3H</td>
<td>X</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3I</td>
<td>XX</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3K</td>
<td>XXX</td>
<td>XXX</td>
<td>X</td>
<td>XXX</td>
<td></td>
</tr>
</tbody>
</table>

#### Number of hits out of 3 replicates

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Hits out of 3 replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>POLR2A</td>
<td>3 of 3</td>
</tr>
<tr>
<td>POLR2B</td>
<td>2 of 3</td>
</tr>
<tr>
<td>POLR2C</td>
<td>1 of 3</td>
</tr>
<tr>
<td>POLR2D</td>
<td>0 of 3</td>
</tr>
</tbody>
</table>

#### Excellent subunit recovery across CMV deletion series

Overall recovery out of all possible subunits.
## Comparison with FLAG-POLR2H

<table>
<thead>
<tr>
<th>Acronym</th>
<th>HT-POLR2H</th>
<th>HT Control</th>
<th>FLAG-POLR2H</th>
<th>FLAG Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>POLR1A</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
<td>XX</td>
</tr>
<tr>
<td>POLR1B</td>
<td>XXX</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPAC1</td>
<td>XXX</td>
<td></td>
<td></td>
<td>XX</td>
</tr>
<tr>
<td>POLR1D</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR1E</td>
<td>X</td>
<td></td>
<td></td>
<td>XX</td>
</tr>
<tr>
<td>hRPA34</td>
<td>X</td>
<td></td>
<td></td>
<td>XX</td>
</tr>
<tr>
<td>TWISTNB</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZNRD1</td>
<td>XX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR2A</td>
<td>XXX</td>
<td></td>
<td>XXX</td>
<td>X</td>
</tr>
<tr>
<td>POLR2B</td>
<td>XXX</td>
<td></td>
<td></td>
<td>XXX</td>
</tr>
<tr>
<td>POLR2C</td>
<td>XXX</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>POLR2D</td>
<td>XXX</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>POLR2E</td>
<td>XXX</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>POLR2F</td>
<td>XX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR2G</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR2H</td>
<td>XXX</td>
<td></td>
<td></td>
<td>XXX</td>
</tr>
<tr>
<td>POLR2I</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR2J</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR2K</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR2L</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3A</td>
<td>XXX</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>POLR3B</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3C</td>
<td>XXX</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>POLR3D</td>
<td>XXX</td>
<td></td>
<td></td>
<td>XX</td>
</tr>
<tr>
<td>POLR3E</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3F</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3G</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3G-LIKE</td>
<td>XX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3H</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3I</td>
<td>XX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3J</td>
<td>XX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POLR3K</td>
<td>XXX</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

- 0 of 3
- 1 of 3
- 2 of 3
- 3 of 3

- Improved recovery with HaloTag
- Higher reproducibility
- Lower background

Imaging of HaloTag®-POLR2H

Co-localization with Endogenous POLR2A

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

Oriented Capture of HaloTag-POLR2H on HaloLink™ Arrays

- **Lyse**
- **Spot** (No purification)
- **Wash**
- **Detect** (Antibodies)

**HaloLink™ Slide Surface** (PEG linker with Chloroalkane)

- **HaloTag-POLR2H Capture on Slide**
  - Experimental (Halo-POLR2H)
  - Control (HaloTag)
  - **Antibodies**
    - POLR2A (CTD)
    - 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.

Capture of Eukaryotic Ribosomes Using S9-HT

**S9-HT Pull-down**

- HaloLink™ Resin
- TEV Cleavage
- SDS Elution
- 60S
- 40S
- Proteins

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

**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
  - 28S
  - 18S
  - 5.8S
  - 5S

• **Purified the 80S ribosome with single step from HeLa cells.**

• **Efficiently cleave complex from resin with TEV protease.**
**Isolated Ribosomes are Bound to mRNAs**

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

---

**Plexor qPCR GAPDH cRNA Amplification**

- RFU
- Cycle

**Total GAPDH cDNA**

- (ng) cDNA

---

**Graphs:**

- S9-HT Ribosome
- HT Control
- S9-HT Ribosome
- HT Control
Isolated Ribosomes are Active for Translation

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

- 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

<table>
<thead>
<tr>
<th>Serum Starve</th>
<th>Pulse</th>
<th>Serum Recovery</th>
<th>Chase</th>
<th>Wash</th>
<th>Image Overlay</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPS9-HT</td>
<td>TMR Label</td>
<td>Green Label</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

- PET
- near IR
Summary

- 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