Labeling Biomolecules with Fluorescent Dyes

Edward Leber
Molecular Probes® Invitrogen™
Eugene, OR.
Outline

• Dyes
• Qdot® Nanocrystals
• Tandem dyes
• Reactive dyes
• Click chemistry
• Antibody and protein labeling kits
**Molecular Probes® Fluorescent Labels**

**Organic Dyes and Labels**

**Alexa Fluor® Dye Series**
- UV to near IR
- Sulfonated for increased H₂O solubility
- Bright, photostable & high QY
- Simply the best
- Spectrally matched to generic dyes (FITC, Cy-dyes)

**Pacific® dyes**
- Optimized for 405 nm or violet laser excitation

**Biotin**
- Can be used with streptavidin conjugates including R-PE, Alexa Fluor® dyes and QDot nanocrystals

**Quantum Dot Nanotechnologies**

**Qdot® Nanocrystals**
- Single excitation
- Uniform size
- Extremely high extinction coefficient

**Phycobiliproteins & Tandems**

R-PE, APC and R-PE-Alexa Fluor® and APC-Alexa Fluor® tandems
- Extremely bright
- Very high extinction coefficient
How to Choose the Optimal Fluorophore

• Know Thy Instrument’s Limitations
  – Excitation source(s) and detection filters

• Know Thy Fluorophore’s Limitations
  – Excitation/emission maxima
  – Extinction coefficient

• Know thy Target

• Use the Spectraviewer

www.invitrogen.com/spectraviewer
## Alexa Fluor® Family members

<table>
<thead>
<tr>
<th>Dye</th>
<th>EC</th>
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<td>AF350</td>
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<td>196000</td>
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<td>AF750</td>
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Alexa Fluor® Dyes Produce Better Conjugates

Comparison of Alexa Fluor® conjugates to other commercially available fluorescent conjugates
Quantum Dots?

Highly fluorescent, nanometer-size, crystals of semiconductor materials

Size of the nanocrystal determines the color
Size is tunable from ~2-10 nm (± 3%)
Size distribution determines emission band width
SpectraViewer showing Quantum Dot Spectra
Tandem - Fluorescence Resonance Energy Transfer (FRET) Dyes

- Emission of one dye overlaps the excitation of a second.
- When close, energy is transferred to the second.
- The second emits a longer wavelength of light than the first.
Alexa Fluor® Tandems – Energy Transfer Fluorophores

- R-PE
- APC
- PE-AF 610
- PE-AF 647
- PE-AF 680
- APC-AF 680
- APC-AF 700
- APC-AF 750

Wavelengths:
- 488 nm
- 633/647 nm
Reactive Dye Chemistries

- Amine labeling
- Thiol labeling
- Click Chemistry
- Groups Other than Thiols or Amines
  - Aldehyde/Ketone labeling
  - Carbodiimide-activated derivatization of carboxylic acids
  - Modifying alcohols
Why Amine Reactive Dyes

• Primary choice for labeling proteins and other biomolecules.

• Wide selection of chemistries and dyes.

• Can produce very stable conjugates.

• Available in easy to use kits.
Amine Reactive Probes

- Succinimidy esters (SE, NHS, SDP esters)
- Trifluorophenyl esters (TFP, STP)
- Isothiocyanates
- Sulfonyl chlorides
- Others
  - OPA (ortho-pthalaldehyde)
  - NDA (naphthalenedicarboxaldehyde)
  - ATTO-TAG, CBQCA
  - NBD chloride (4-chloro-7-nitrobenzofurazan)
  - Dichlorotriazines (5-DTAF)
Amine Reactive Dyes: Succinimidyl Esters

protein NH₂ + Succinimidyl ester, aka SE, NHS ester, N-hydroxysuccinimidyl ester

Amide bond is formed
Amine Reactive Dyes: Isothiocyanates

 Isothiocyanates, aka ITC

\[ \text{protein} \quad \text{NH}_2 + S-C=N \quad \text{fluorescent label} \]

pH \sim 9

\[ \text{protein} \quad N-C=N \quad \text{fluorescent label} \]

thiourea bond
Amine Reactive Dyes: TFP and STP Esters

protein + NH₂ + fluorescent label → protein

TFP esters (X=H)
STP esters (X=S0₃H)

amide bond

+ X

H

OH

FF

FF

FF

FF

FF

FF
Amine Reactive Dyes: Sulfonyl Chlorides

Sulfonyl chlorides

protein $\text{NH}_2$ + Cl$\text{S}$

pH $\sim$ 9

protein $\text{NH}$ $\text{SO}$ $\text{O}$

Sulfonamide bond
Why Thiol Reactive Dyes

• Free thiols are rare in bio-molecules allowing thiols to be site specific labels when combined with site selective mutations.

• Many natural thiols are in environmentally sensitive pockets within proteins making them good sites for functional reporters.

• Thiols need to be in reduced form to react.
Thiol Reactive Dyes

- Maleimide
- Iodoacetamide
- Methyl bromide
- Others
  - Environmentally sensitive thiol reactive naphthalene derivatives
    - Acrylodan, IAEDANS, IAANS, MIANS
  - Bimanes
    - Monobromobimane
Thiol Reactive Dyes: Maleimides

protein + Maleimides → fluorescent label → thioether bond → protein
Thiol Reactive Dyes: Iodoacetamides

protein SH + Iodoacetamide → fluorescent label

protein S-thi ether bond fluorescent label

thioether bond
Other reactive chemistries: Aldehyde/Ketone Modification

\[ \text{protein, oligo, carbohydrate} + \text{fluorescent label} \]

Where:
- \( X=\text{H} \) Aldehyde
- \( X=\text{alkyl} \) Ketone

Hydrazides, hydrazines, semi-carbazides

Hydrazone bond
Other Reactive Chemistries: Oxidation of a Sugar Residue to an Aldehyde

\[ \text{O}_{2} \xrightarrow{\text{Galactose Oxidase}} \text{H}_{2}\text{O}_{2} \]

\[ \text{R} \rightarrow \text{glycolipid, polysaccharide or glycoprotein} \]
Other Reactive Chemistries: Carbodiimide Modification of Protein Carboxylic Acids

\[ \text{Carbodiimide} \rightarrow \text{Protein} - \text{COOH} \rightarrow \text{Protein} - \text{CO} - \text{C} - \text{NHR}^1 \rightarrow \text{Protein} - \text{C} - \text{N} - \text{C} - \text{NHR}^2 \]
Click chemistry fills a void in detection techniques, where researchers currently use methods that are either harmful or inadequate.

- Small, biologically unique and inert functional groups
- Reacts quickly and exclusively
- Mild reaction conditions
- Use with *any readout* (fluorescence, absorbance, chemiluminescence)
- Use on *any platform* (blot, gel, flow cytometer, fluorescence microscope, high-content imager, mass spectrometer, etc.)
- Unprecedented sensitivity (as low as femtomole or one quadrillionth)
What is “Click” Chemistry?

Click chemistry is a concept introduced by K. Barry Sharpless in 2001 and describes chemistry tailored to generate substances quickly and reliably by joining small units together as nature does.

\[
\text{Azide} \quad \begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N}^+ \\
\text{N}^-
\end{array} + \quad \text{DNA} \quad \rightarrow \quad \text{Triazole}
\]

Alexa Fluor® 488 Dye

15-30 minutes

Copper catalyzed azide-alkyne cycloaddition
Click-iT® Applications: DNA Synthesis

Anti-BrdU antibody

Inaccessible without denaturation

Incorporated BrdU

Click-iT™ Alexa Fluor® azide

Accessible

Incorporated EdU
Click-iT® Applications: Glycoproteins

Ac$_4$ManNAz

Ac$_4$GalNAz

Ac$_4$GlcNAz

Feed cells 1–3 days

Cell surface sialic acids

O-linked cell surface and intracellular O-GlcNAc

Intracellular O-GlcNAc


Dube DH and Bertozzi CR; Curr Opin Chem Biol. 2003; 7(5) 616-625
• Introduction of an azidohomoalanine
• First non-radioactive alternative to $^{35}$S-methionine
• Incorporation via the cell’s natural enzymatic machinery
• Detection of newly synthesized proteins via click chemistry

Click-iT® Applications: Nascent Proteins Synthesis

1. Metabolic incorporation
2. Click-iT™

azidohomoalanine

methionine

detection of newly Synthesized proteins
Antibody & Protein Labeling Kits
Do You Need a Directly Labeled Conjugate?

- For use with “higher” abundance targets
- To reduce background due to non-specific secondary detection
- For use with multiplexed analyses with same-species antibodies

**Directly-Labeled Primary Antibody**
- Low background
- Covalent attachment of label via amine-reactive dyes to lysines
- Labeling could affect antigen recognition site
- Fluorescent signal similar to Zenon®-Labeled Antibody

**Zenon®-Labeled Antibody**
- Low background
- Non-covalent attachment of Zenon® fragment to Fc portion
- Labeling will NOT affect antigen recognition site
- Fluorescent signal similar to Direct-labeled Primary antibody

**Indirectly-Labeled Secondary Antibody or Streptavidin**
- Higher Background
- Brighter Signal
Labeling an Antibody

**Organic Dyes:** >1 dye/antibody

**R-PE & Tandems:** 1 dye/antibody

**Qdot® Nanocrystals:** >1 antibody/Qdot® Nanocrystal
**Antibody & Protein Labeling Kits**

<table>
<thead>
<tr>
<th>APEX Antibody Labeling Kits</th>
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<tbody>
<tr>
<td>Microscale Protein Labeling Kits</td>
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<tr>
<td>Monoclonal Antibody Labeling Kits</td>
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<tr>
<td>Protein Labeling Kits</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SAVI™ Protein / Antibody Labeling Kits</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Covalent label attachment</td>
</tr>
<tr>
<td>* HCL optimized for SAVI™ Applications</td>
</tr>
<tr>
<td>* HCL control reagent</td>
</tr>
</tbody>
</table>

**Applications**

- Flow Cytometry
- Microscopy
- ICC
- IHC

**Available Labels**

- Alexa Fluor® Dye Series
- Biotin and DSB™X biotin
- Oregon Green® Dye
- Pacific™ Dye
- Standard Dyes (fluorescein)

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<table>
<thead>
<tr>
<th>Zensa® Antibody Labeling Kits</th>
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<tbody>
<tr>
<td>6 minute antibody labeling</td>
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<tr>
<td>* Non-covalent label attachment</td>
</tr>
<tr>
<td>* Organic dyes &amp; IR-PE</td>
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</tbody>
</table>

**Applications**

- Flow Cytometry
- Microscopy
- ICC
- IHC

**Available Labels**

- Alexa Fluor® Dye Series
- Alexa Fluor® tandem
- Phycobiliproteins (R-PE, APC)
- Biotin and DSB™X biotin
- Oregon Green® Dye
- Pacific™ Dye
- Standard Dyes (fluorescein)

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<table>
<thead>
<tr>
<th>QD® Nanocrystal Secondary Antibody Labeling Kits</th>
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<tbody>
<tr>
<td>* Covalent label attachment</td>
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</table>

**Applications**

- Flow Cytometry
- Microscopy
- ICC
- IHC

**Western blots**

SAVI™ Applications

**Available Labels**

- QD® Nanocrystals
- 525, 565, 585, 605, 635, 705, 800

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* Requires specific primary antibodies to target or short incubations (4h)

* Verify exam resources
Picking an Antibody Labeling Kit

1. What label do you want?
2. How much antibody do you want or need to label?
3. Where do I find this information?
4. Should the label be covalent or non-covalent?
5. Does the antibody contain stabilizing proteins?
1. What Label do You Want?

Dye selection guide for protein labeling kits

<table>
<thead>
<tr>
<th>Flow cytometry imaging</th>
<th>Emission color</th>
<th>Fluorophore</th>
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For an interactive version visit: www.invitrogen.com/spectraviewer
## 2. How Much Antibody Do You Want or Need To Label?

<table>
<thead>
<tr>
<th>Amount IgG</th>
<th>Product</th>
<th>Key features</th>
</tr>
</thead>
</table>
| <1–20 μg   | Zenon® IgG Labeling Kit                | • Labeled IgG antibodies ready to use in 10 min  
• Isotype-specific labeling  
• Fast, noncovalent attachment of label  
• Labeling compatible with stabilizing proteins such as BSA |
| 10–20 μg   | APEX Antibody Labeling Kit             | • Labeled IgG antibodies ready to use in 2.5 hr (~15 min hands-on time)  
• Covalent attachment of label  
• Labeling compatible with stabilizing proteins such as BSA |
| 20–100 μg  | Microscale Protein Labeling Kit        | • Labeled antibodies ready to use in 2 hr (~30 min hands-on time)  
• Covalent attachment of label  
• Optimized for proteins between 10–150 kDa, including IgG antibodies (~150 kDa)  
• Stabilizing proteins must be removed from sample before labeling |
| 100 μg     | Monoclonal Antibody Labeling Kit       | • Labeled IgG antibodies ready to use in 90 min (~15 min hands-on time)  
• Covalent attachment of label  
• Optimized for IgG antibodies (~150 kDa)  
• Stabilizing proteins must be removed from sample before labeling  
• Designed to label polyclonal and monoclonal IgG antibodies |
| 1 mg       | Protein Labeling Kit                   | • Labeled antibodies ready to use in 2 hr (~30 min hands-on time)  
• Covalent attachment of label  
• Optimized for IgG antibodies (~150 kDa)  
• Stabilizing proteins must be removed from sample before labeling |
| 0.3–5 mg   | SAIVI™ Antibody Labeling Kits          | • Labeled IgG antibodies ready to use in 90 min (~10 min hands-on time)  
• Covalent attachment of label  
• Optimal degree of labeling for *in vivo* imaging applications  
• Labeled antibodies ready for use in applications that require azide-free reagents, such as live-cell imaging or direct injection into animals |
3. Where Do I Find This Information?

- www.invitrogen.com/ablabeling

How Much Antibody Would You Like to Label?

- < 1-20 µg
  - Zenon® Antibody Labeling Kits
    - Learn More about Zenon® Antibody Labeling Kits

- 10-20 µg
  - APEX Antibody Labeling Kits
    - Learn More about APEX Antibody Labeling Kits

- 20-100 µg
  - Microscale Antibody Labeling Kits
    - Learn More about Microscale Antibody Labeling Kits

- 100 µg
  - Monoclonal Antibody Labeling Kits
    - Learn More about Monoclonal Antibody Labeling Kits

- 1 mg
  - Protein Labeling Kits
    - Learn More about Protein Labeling Kits

- 0.5-3 mg
  - Small Animal in Vivo Imaging (SAIVI™) Antibody Labeling Kits
    - Learn More about Small Animal In Vivo Imaging (SAIVI™) Antibody Labeling Kits
4. Should the Label be Covalent or Non-covalent?

**Covalent attachment**

These conjugates are stable for **months** to even **years**...

Can be used with any antibody application

**Products:**
- APEX Antibody Labeling kits
- Microscale protein labeling kits
- Monoclonal antibody labeling kits
- Protein labeling kits

**Non-Covalent label attachment**

Conjugates are stable for **hours** to **maybe days**...

Recommended for use with applications requiring short incubations

**NOT recommended** for immunohistochemistry (IHC) applications requiring several hours to overnight antibody incubations

**Products:**
- Zenon® Antibody Labeling Kits

Label directly & covalently attached to the IgG antibody

Label non-covalently attached to the IgG antibody using directly labeled anti-FC fab fragments
5. Does the Antibody Contain Stabilizing Proteins?

Typically:

- The stabilizing is bovine serum albumin (BSA)
- This is used to stabilize small amounts of packaged IgG antibodies
- Whether the IgG contains BSA will either be listed on the vial or in the product data sheet

**So, why should I care?**

If they are labeling with a non-Zenon® kit (i.e., microscale, monoclonal & protein) these kits utilize amine-reactive dyes that will react with the stabilizing proteins in addition to the IgG antibody. *If they are not using Zenon and the stabilizing proteins are not removed, their antibody will be underlabeled and produce a very weak signal.*

Zenon® antibody labeling kits are completely compatible

**If stabilizing proteins are present, it’s not the end of the world...**

All of the non-Zenon® antibody labeling kits include simple protocols to remove the stabilizing proteins as long as there is ~0.5 mg IgG
**Zenon® vs non-Zenon® Antibody Labeling Kits**

**Zenon® Antibody Labeling**
- Fastest way to label IgG (10 minutes!!)
- Very, very easy-to-use
- Perfect for small quantities of IgG (<20 mg)
- Great way to attach, Alexa Fluor® dyes R-PE, R-PE-tandems (and other fluorescent proteins or enzymes)

**APEX, Microscale, Monoclonal Antibody & Protein Labeling Kits**
- Fast (1.5-2 hours, only 15-30 minutes of actual hands-on time)
- Very easy-to-use
- Perfect for larger quantities of IgG (>20 mg - 1 mg)
- Great way to covalently attach Alexa Fluor® dyes & biotin
~ 1 μg primary antibody

Add Zenon reagent at desired molar ratio

Incubate 5 minutes

Block with relevant IgG & Use
Zenon® Labeling Technology

**Kinetically trapped complex formation: key to making the system work.**

**Fast complex formation**

![Fast complex formation diagram](image1)

**Slow complex dissociation**

![Slow complex dissociation diagram](image2)

**Graph 1**

Mouse anti-biotin IgG₃

+ Alexa Fluor 488 Zenon

Capture by BSA-biotin in microtitre plate

**Graph 2**

Blocked mouse anti-biotin IgG₃

+ Alexa Fluor 488 Zenon

Capture by BSA-biotin in microtitre plate

**Fluorescence**

**Time (minutes)**

**Fluorescence (% of initial)**

**Time (hours)**
Why Use Zenon® Antibody Labeling

**Simple**
No need to use secondary antibodies anymore

**Speed**
Zenon labeling complexes are ready to use for cell staining within 5 minutes…

**Quantitative Labeling**
100% of the primary antibody sample is labeled.

**No Preparation**
Removal of exogenous proteins such as serum albumin from primary antibody samples is unnecessary.

**Compatibility**
Multiple Zenon One–labeled mouse antibodies can be used in the same immunolabeling protocol.

**Economy**
A standard Zenon labeling requires only 1 µg of primary antibody (versus 20 µg for chemical labeling). Saves time and money.

Visit: [www.invitrogen.com/Zenon](http://www.invitrogen.com/Zenon)
APEX Antibody Labeling Kits

- Convenient: APEX Kits label small amounts of antibody, 10-20 μg
- Covalent attachment of label: Once attached, label will not come off
- Compatible: Stabilizing proteins (i.e., BSA) or amine-containing buffers (i.e., TRIS) will not interfere with labeling

Customer requirements:

- 10-20 μg IgG antibody
- Standard pipette (for 200 μL volume)
- ~15 minutes “hard labor” (2.5 hours total)

With pipette, apply to APEX labeling tip
- Apply IgG antibody
- Fluorescent label
- Wash buffers

Multiple steps: Customer will need to either apply or elute
- Wash buffers
- Labeled IgG

With pipette, elute
- Wash buffers
- Labeled IgG
# Antibody & Protein Labeling Kits

## APEX Antibody Labeling Kits
- Microscale Protein Labeling Kits
- Monoclonal Antibody Labeling Kits
- Protein Labeling Kits

### Applications
- Flow Cytometry
- Microscopy
  - ICC
  - IHC

### Available Labels
- Alexa Fluor® Dye Series
- Biotin and DSB™-X biotin
- Oregon Green® Dyes
- Pacific™ Dyes
- Standard Dyes (fluorescein)

## SAIVI™ Protein / Antibody Labeling Kits
- Covalent label attachment
- HCl optimized for SAIVI™ Applications
- HCl control reagent

### Applications
- Small Animal in-vivo Imaging (SAIVI™) Applications

### Available Labels
- Near IR Alexa Fluor® Dyes
  - Alexa Fluor® 647, 680 & 750

## ZenoX® Antibody Labeling Kits
- 6 minute antibody labeling
- Non-covalent label attachment
- Organic dyes & IR-PE

### Applications
- Flow Cytometry
- Microscopy
  - ICC
  - IHC

### Available Labels
- Alexa Fluor® Dye Series
- Alexa Fluor® tandem dyes
- Phycocerythrin (PE, APC)
- Biotin and DSB™-X biotin
- Oregon Green® Dyes
- Pacific™ Dyes
- Standard Dyes (fluorescein)

## QDent® Nanocrystal Secondary Antibody Labeling Kits
- Covalent label attachment

### Applications
- Flow Cytometry
- Microscopy
  - ICC
  - IHC
- Western blots

### Available Labels
- QDent® Nanocrystals
  - 525, 565, 635, 685, 635, 705, 600

* Requires optimized primary antibodies to target or short incubations [4 h]

* Verify assay resources
## Antibody & Protein Labeling Kits Overview

<table>
<thead>
<tr>
<th>Kit</th>
<th>Sample Requirements</th>
<th># of Rxns</th>
<th>Covalently Attached Label</th>
<th>Total Time</th>
<th>Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zenon® IgG Labeling Kit</td>
<td>&lt;1 μg to 20 μg of IgG antibody</td>
<td>10-50</td>
<td>No</td>
<td>10 minutes</td>
<td>• Labels small amounts of IgG, very fast – 5 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Can label in serum or amine-containing buffers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Isotype specific labeling (IgG&lt;sub&gt;1&lt;/sub&gt;, IgG&lt;sub&gt;2a&lt;/sub&gt;, etc)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Label with phycobiliproteins (R-PE) and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>phycobiliprotein-tandems (Alexa Fluor® 610-R-PE) as well as “classic”</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>organic dyes, ie, Alexa Fluor® dyes and fluorescein (FITC)</td>
</tr>
<tr>
<td>APEX Antibody Labeling Kit</td>
<td>10 μg to 20 μg of IgG antibody</td>
<td>5</td>
<td>Yes</td>
<td>2.5 hours</td>
<td>• Labels small amounts of IgG easily</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(15 min.*</td>
<td>• Can label antibodies in serum or amine-containing buffers</td>
</tr>
<tr>
<td>Microscale Protein Labeling Kit</td>
<td>20-100 μg of protein or IgG antibody</td>
<td>3</td>
<td>Yes</td>
<td>2 hours</td>
<td>• Optimized for proteins, ~10-150 kDa, including IgG Antibodies</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(30 min.*</td>
<td></td>
</tr>
<tr>
<td>Monoclonal Antibody Labeling Kit</td>
<td>100 μg of IgG antibody</td>
<td>5</td>
<td>Yes</td>
<td>1.5 hours</td>
<td>• Optimized for IgG antibodies, MW ~150 kDa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(15 min.*</td>
<td>• Can label monoclonal or polyclonal antibodies</td>
</tr>
<tr>
<td>Protein Labeling Kit</td>
<td>1 mg of IgG</td>
<td>3</td>
<td>Yes</td>
<td>2 hours</td>
<td>• Optimized for IgG antibodies, MW ~150 kDa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(30 min.*</td>
<td></td>
</tr>
<tr>
<td>QDot® Nanocrystals Labeling Kits</td>
<td>0.3 mg of IgG antibody</td>
<td>2</td>
<td>Yes</td>
<td>4 hours</td>
<td>• Optimized for IgG antibodies</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.5-2 hours*</td>
<td>• QDot® IgG conjugates</td>
</tr>
</tbody>
</table>

* Hands on time
How These Protein / Antibody Labeling Kits Work

Target amine must be deprotonated, $:\text{NH}_2$ to react with an activated ester.

Increasing the pH (:OH) of the reaction solution will deprotonate the amines from $\text{NH}_3$ the majority of the species at physiological pH (pH 7) to $:\text{NH}_2$

After a very fast reaction, conjugate is purified via size exclusion chromatography.

Activated esters produce the most stable conjugates.
Applications for Labeled Antibodies

Antibodies are a type of protein that are designed to recognize and bind specific antigens.

The types of antigens that antibodies can bind to are quite varied and include other proteins, peptides, carbohydrates, lipids, nucleic acids or small molecules.

Antibodies are frequently used for identification, localization and quantification of specific antigens in

- Biomedical Research
- Clinical Research
- Diagnostics

Directly labeled antibodies permit multiplexed analyses and are compatible with a wide variety of instrument platforms.
Protein & Monoclonal Antibody Labeling Kits

Optimized for Direct IgG Labeling
- Simple and easy to use protocols
- Reactive dye, buffers, and purification components provided
- Reactive dye is pre-measured
- Optimized for labeling IgG (145,000) though other proteins can be labeled with MW >30,000 (though not recommended) as a function of purification resin cutoff of >30,000 Da.

- **Alexa Fluor Protein Labeling Kits**
  - Requires ~1 mg of purified IgG
  - 3 reactions

- **Alexa Fluor® Monoclonal Antibody Labeling Kit**
  - Requires ~100 micrograms of purified IgG
  - 5 reactions
  - Can label monoclonal and polyclonal IgG antibodies
Alexa Fluor® Protein Labeling Kits

1. Add bicarbonate to the protein solution
2. Add protein to the reactive dye
3. Incubate 15 minutes
4. Purify the conjugate on the column
Alexa Fluor® Monoclonal Antibody Labeling Kits

1. Add bicarbonate to the protein solution
2. Add protein to the reactive dye
3. Purify the conjugate with the spin column

Incubate one hour
Microscale Protein Labeling Kits

Optimized for Direct IgG and Proteins >12,000 Dalton Labeling

- Simple and easy to use protocol
- Reactive dye, buffers, and purification components provided
- Customers calculate molar ratio of dye:protein
- Requires 20-100 mg of purified protein or antibody
- Yields 60-90%
Conjugating Antibodies to Qdot® Nanocrystals

- Convert amines on Qdot® Nanocrystals to thiol reactive maleimides
- Reduce disulfides on antibody to free thiols
- Crosslink Qdot® Nanocrystals to antibodies by reacting maleimides with free thiols
- Purify conjugate
Conjugating Antibodies with QDot® Nanocrystals

- Quantum Dot Activation, 1 hr
- Removal of Excess SMCC via Size Exclusion Column
- React, 1 hr
- Quench, 0.5 hr
- Concentrate via Ultrafiltration (~0.5 hr)
- Purify via Size Exclusion Column (~0.5 hr)
- Total Time: 4-5 hrs

- Not for the novice
- Do you need a QDot® nanocrystal? – would a standard fluorophore do?
QDot Nanocrystal Antibody Conjugation Hints

- **Start with your antibody at the highest concentration:**
  - 5-10 mg/ml concentration at the start is better than the 1 mg/ml concentration
  - conjugation will be more efficient

- **For monoclonal antibody, the reduction conditions may need to be tailored to the specific antibody being used**
  - check the extent of reduction before doing a full scale conjugation
  - reduce a small sample and run it on a NAP-5 column to get rid of the DTT
  - then use Ellman's reagent as a colorimetric assay for free sulphydryl groups (we can provide a protocol for this). 4 to 10 free sulfhydryls, the conjugation should work
  - if every disulfide in the Ab gets reduced, you won't get a functional conjugate, so err on the side of less DTT rather than more

- **Coordinate the SMCC modification and antibody reduction reactions so the reduced antibody doesn't sit after coming off the column**
  - it should be mixed with the SMCC-modified dots immediately after it comes off the column.

- **When collecting the final conjugate from the separation (Superdex-200) column**
  - make sure you **start collecting drops as soon as colored material appears** at the column tip
  - **collect no more than ten drops**, no matter how much material you think you are giving up
THANK YOU!

Questions?
techsupport@lifetech.com
800.955.6288
Labeling Biomolecules with Fluorescent Dyes

Molecular Probes® Invitrogen™
Eugene, OR.