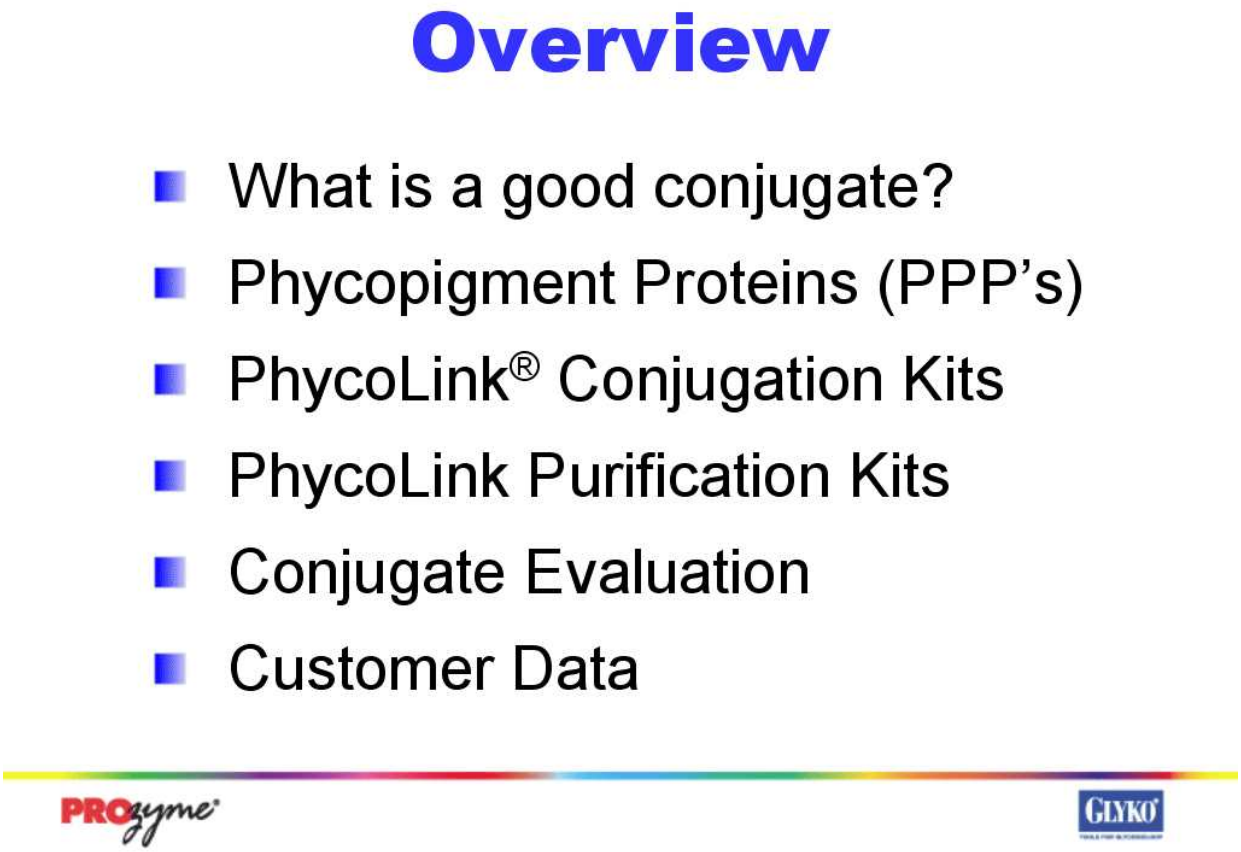
NOTE: Reflects some changes from original (presented at ISAC XXIII) in order to provide a stand-alone presentation for first-time viewers.

Slide 1 - Introduction

Welcome. Thank you all for being here. I assume you're here because you'd like to learn more about using PhycoLink® Conjugation and Purification Kits to make your own conjugates. We'd like to start by thanking a long-time customer for giving us our title when he told us that our kits "just make the best DARN conjugates." We simply couldn't have said it any better ourselves.

Many of you know ProZyme as the manufacturer of phycobiliproteins (*e.g.* RPE, APC, *etc.*) sold under the PhycoPro™ tradename, which are widely used in flow cytometry and other fluorescence applications. You may also be familiar with our PhycoLink line of fluorescent conjugates (antibodies, streptavidin, *etc.*), which are value-added products for use in fluorescence applications.

What we hope to accomplish today is to impart to you some of our experience, accumulated over many years working with a particular class of fluorescent pigments, the phycopigment proteins, and to dispel some of the mystery from what has largely been considered a black box in terms of making conjugates from these fluors.



Slide 2 - Overview

We're going to start out by discussing some of the properties that define a good conjugate. Then we'll consider some of the characteristics of the phycopigment proteins (PPP's). I need to stop to define this term. You're probably familiar with the class of fluorescent molecules known as the phycobiliproteins, large, water-soluble proteins derived from cyanobacteria and eukaryotic algae, having absorbance and emission wavelengths in the visible range; examples include RPE and APC. With our recent licensing of the PerCP pigment protein from Becton, Dickinson and Company, Inc., in order to be inclusive/correct we've had to expand this term. We'll now refer to this class of molecules as the phycopigment proteins, PPP's or three P's—I'll use these terms interchangeably throughout the presentation.

Next, we'll talk about using our PhycoLink Conjugation Kits to make conjugates from these molecules. Then, we'll consider some of the scenarios for which it may be desirable to utilize our PhycoLink Purification Kits in order to purify the conjugate you've made. I'd like to mention at this point that I'm not going to read our protocols to you. All of the procedures and supporting figures/data to which I'll refer in the course of today's presentation are available on our website for you to review at your leisure. Also, we've recently rewritten all of our conjugation kit booklets in order to remove any ambiguity in cases where we could have been more clear. So, for those of you who may not have

worked with one of our kits in a while, I encourage you to download the revised versions of the booklet. Just click into the PPP of choice to find the product insert (booklet), FAQs, TechNotes, *etc.*

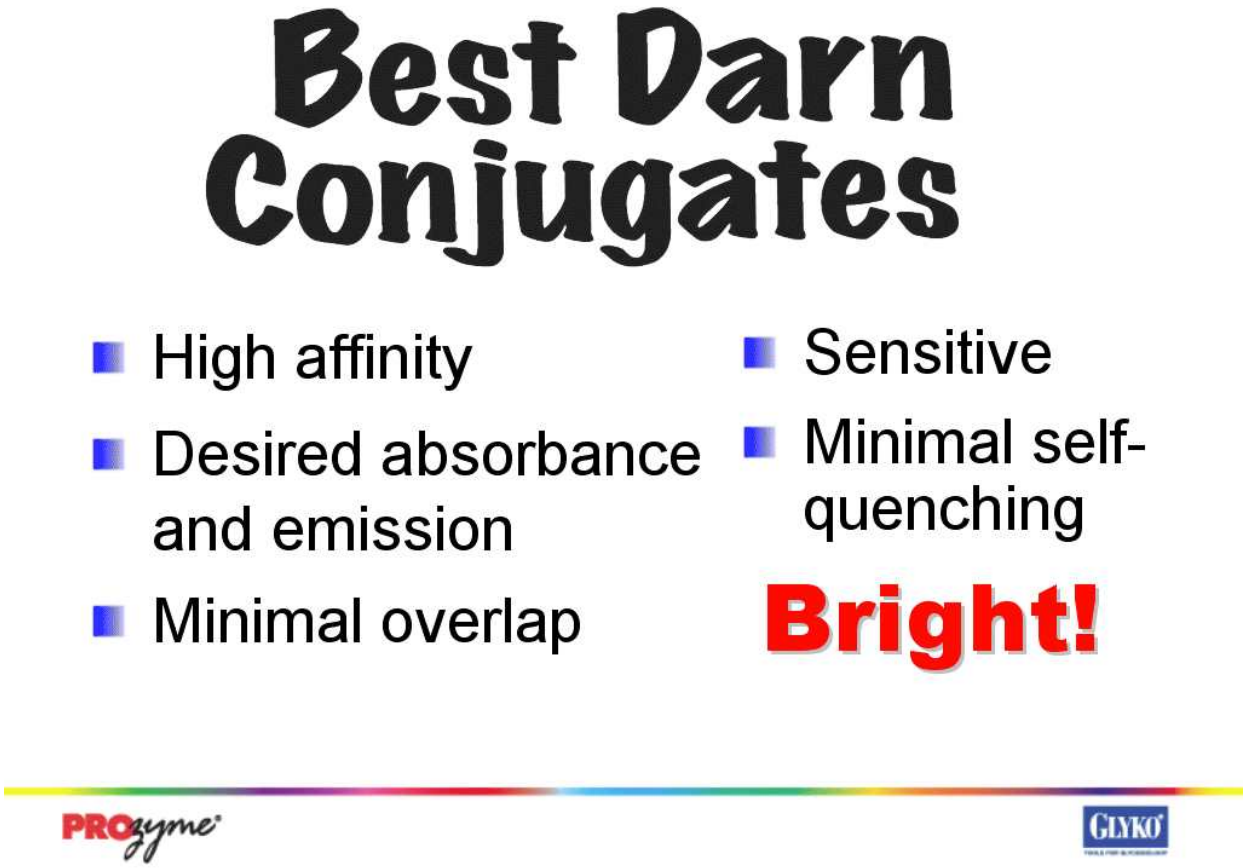
http://www.prozyme.com/phycolink/pj-kits.html

Then, we'll discuss some tools for evaluating conjugates, which are equally applicable to conjugates you have made using our kits as well as those purchased from commercial sources. Lastly, if time permits, I'd like to close by showing examples of customer-furnished data which illustrates the utility of PPP conjugates in applications including flow cytometry, fluorescence microscopy and imaging cytometry.

Slide 3 - Why make your own?

I assume you're here because you either need or want to make your own conjugates, possibly for one or more of these reasons:

- Want a direct conjugate in order to avoid two-step staining.
- Antibody unavailable in the color you want.
- Have only limited quantities of antibody to work with (maybe you're making your own, or it's expensive to buy purified antibody).
- Need a bright tag for a dim (*i.e.* low affinity/abundance) marker.
- Cost-effective alternative to purchasing commercial conjugates, especially if you use a lot of the same conjugate (*e.g.* as a gating reagent in every tube). Also, custom conjugations are expensive and usually require a significant amount of antibody (≥5 mg)!!!



Slide 4 - Best Darn Conjugates

Now, let's consider some of the features that we want in that best darn conjugate:

- High affinity - the binding of your target protein to its cognate ligand should not be compromised as a result of the conjugation process. For example, if your protein is an antibody, the antigen binding site should not be obstructed by the label.

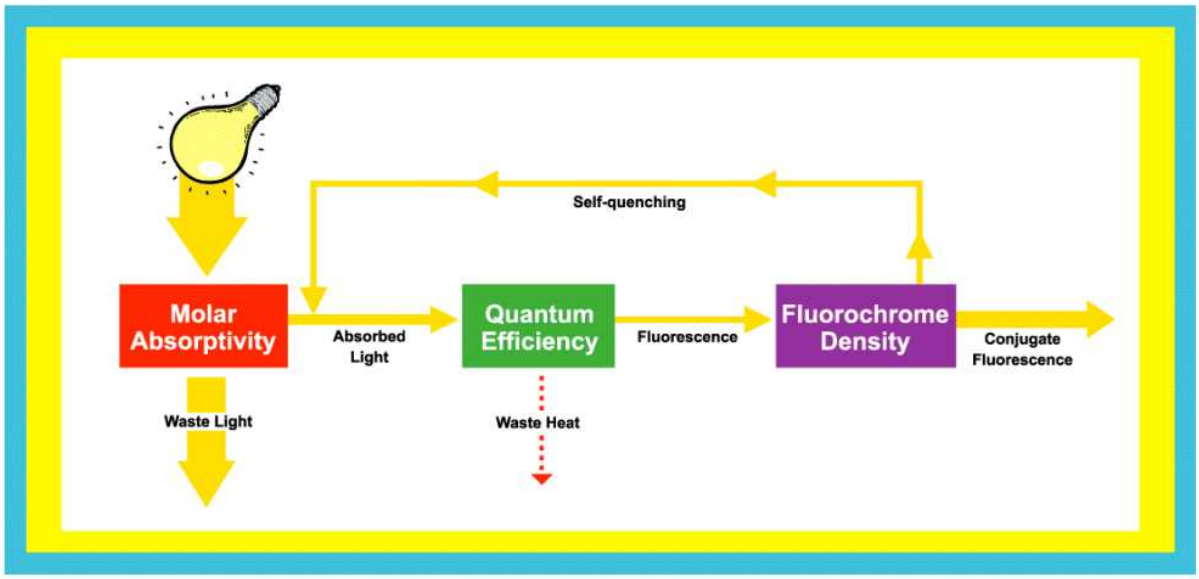
- Desired absorbance/emission & minimal overlap - usually you have predefined parameters in mind with regard to absorbance and emission wavelengths to ensure compatibility with your particular instrument as well as to minimize spectral overlap with other colors in your panel; especially important when doing multi-color (or to use the latest term, "polychromatic") analysis.

- Sensitive - the signal should be robust over a range of concentrations, and have low background.

- Minimal self-quenching - the signal should remain proportional to concentration over a range of concentrations and antigen expression levels.

- Bright - and what we overwhelming hear from customers is that the best darn conjugates must be bright!

Conjugate Brightness



See our **TechNote TNPJ210**

Slide 5 - Conjugate Brightness

So, let's take some time now to consider the factors that combine to determine any conjugate brightness: molar absorptivity, quantum efficiency, fluorochrome density and finally, self-quenching; the first two and the fourth are characteristics of the fluorochrome and the third is a result of the conjugation process:

- Molar Absorptivity (*aka* Molar Extinction Coefficient). It is a measure of how well a molecule absorbs energy at a given wavelength (usually expressed at its absorbance maximum). In reality, molecules absorb over a range of wavelengths constituting the molecule's absorptivity spectrum, of which we'll see some examples in a moment. Values can range over several orders of magnitude between the least and most absorptive molecules, making this factor of primary importance in determining brightness.

  NOTE: Even fluors with the highest molar absorptivities will miss a large portion of the incident light (waste light in diagram). This is not necessarily a bad thing as it allows us to multiplex.

- Quantum Efficiency. This is the proportion of absorbed photons emitted as fluorescence. It is never 100%, as a portion is always dissipated as heat (note the difference in the thickness of the arrows depicting Absorbed Light *vs*. Fluorescence

in the diagram). Due to poor reproducibility among methods of measurement, it is difficult to compare values. Useful fluors have values between 0.25 - 0.9+, making the maximum difference between the worst and best fluors only 3- to 4-fold. This factor is therefore of secondary importance in determining conjugate brightness, but still worthy of consideration.

- Fluorochrome Density. This is the number of fluorochrome molecules per conjugate molecule. It is often desirable to try to maximize this, but there can be drawbacks. One is decreased solubility. Fluorescein is a good example—too many and a conjugate precipitates.

  [I would like to pause a moment to explain what is actually going on here, since this is one of the most misunderstood concepts about conjugates.

  Many manufacturers would have you believe that their conjugates are 1:1, that is, one ligand is conjugated to one fluorochrome. As a result, customers have an oversimplified picture of what the conjugates actually look like. Although the molar ratio of antibody-to-fluor in the conjugate may be reported as 1:1—and that's probably rarely the case, the conjugate is really a population of many antibody and fluorochrome molecules, say a mix of 1:1, 2:2, 3:3; or even 2:3 and 3:2.

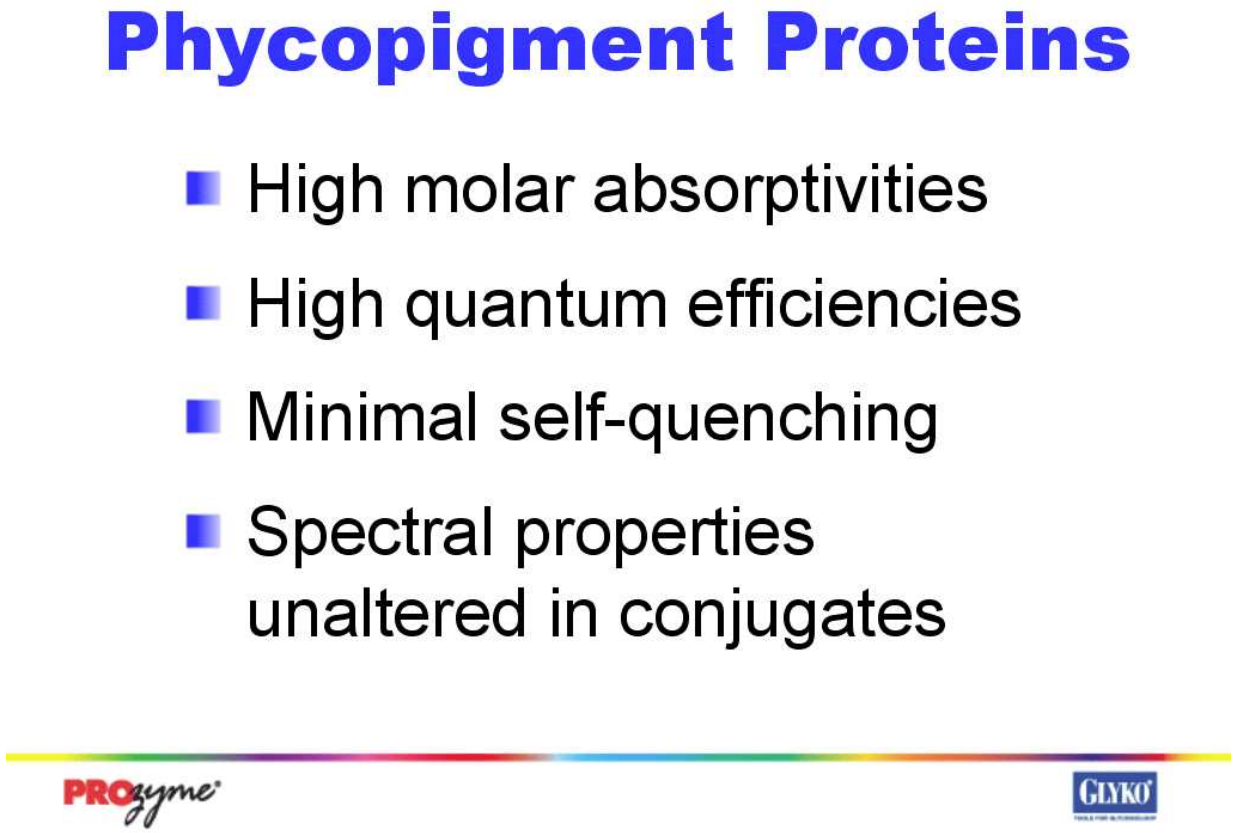
  Only one of these antibody molecules participates per antigen binding event, but what is measured is the cumulative fluorescence of all of the fluorochrome molecules in the conjugate. Per binding event, many fluors are measured, making that very bright conjugate.]

- Self-quenching. Another drawback to increasing fluorochrome density is self-quenching (fluorescein is a good example). Self-quenching can occur when the emission spectrum of a fluorochrome overlaps significantly with its absorbance spectrum, resulting in reabsorption of an emitted photon before it reaches the detector. The result is decreased apparent fluorescence signal, because the probability of a photon being re-emitted is decreased.

  Self-quenching is increased by any process that decreases the randomness of the distribution of molecules in solution, and is most problematic when many small fluorochromes with short Stokes shifts are incorporated into a single conjugate (a strategy commonly used by manufacturers to compensate for low molar absorptivity, quantum efficiency, *etc.*).

See TechNote TNPJ210 for a more exhaustive discussion with representative values:

http://www.prozyme.com/pdf/tnpj200.pdf



Slide 6 - Phycopigment Proteins

Now let's see how the PPP's measure up in terms of these characteristics. The PPP's have optimum spatial chromophore arrangement which optimizes each of these factors:

NOTE: The subunits and their interactions are beyond the scope of this presentation, but the effects can be summed up in terms of the factors we have defined.

- The PPP's have extremely high molar absorptivities. They've evolved to be excellent light antennae; their very survival has depended on their ability to scavenge light from the low-light environments in which their parent organisms live. The PPP's also have a large number of chromophores per molecule (~35 in RPE) *vs.* only one per molecule in small, synthetic dyes (*e.g.* CyDye™ Fluors, Alexa Fluor® dyes, fluorescein, rhodamine, *etc.*). Fluorescein and Rhodamine B have values less than 100 (with units of $x10^3$ $cm^2 \cdot mol^{-1}$). The Alexa Fluor® dyes and CyDye™ Fluors typically have values ranging from about 100 to 250. By contrast, the PPP's are up to several orders of magnitude higher: RPE has a value of nearly 2000, while BPE comes in over 2400 (at its max)!

  I'd like to digress for a moment and mention that, although RPE has traditionally been the phycoerythrin of choice for flow cytometry, with the

introduction/installation of instruments equipped with green lasers (*e.g.* 532 nm), BPE, with its excitation maximum at 545 nm, might be a worthwhile alternative, given its extremely high molar absorptivity.

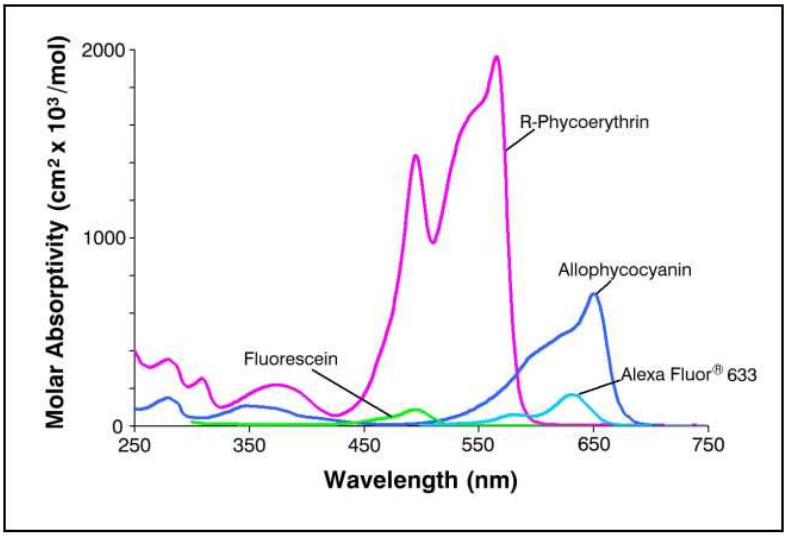
- In terms of quantum efficiencies, the PPP's have been shown to be among the most efficient of all fluors, and for a very good reason: the PPP's are responsible for shuttling light in an energy cascade to chlorophyll in the photosynthetic reaction center through a process known as FRET (fluorescence resonance energy transfer)—the organism's survival depends on this being an efficient process.

- The PPP's exhibit minimal self-quenching; the fluors are distributed optimally along a protein backbone.

  Additionally, PerCP has an extremely long Stokes shift (nearly 200 nm; absorbance 482 nm and emission 675 nm). It would be difficult to rival this, even with a tandem dye!

- Lastly, the PPP's can be conjugated to antibodies or other proteins without alteration of their spectral properties.

Molar Absorptivity



Conclusion: **PPP's** are the **brightest** fluorophores available!

Slide 7 - Molar Absorptivity

This figure provides a comparison of just one of the factors we have been discussing, molar absorptivity. You're probably used to looking at normalized absorption curves, but this is unnormalized data to allow a true comparison of different fluors. As you can see, relative to RPE and APC, Fluorescein and Alexa Fluor® 633 are barely off axis! The conclusion is clear: the PPP's are the brightest fluors available!

Phycopigment Proteins

- R-Phycoerythrin (RPE)
- Allophycocyanin (APC)
- Peridinin-chlorophyll-protein complex (PerCP)
- and more…

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Slide 8 - Phycopigment Proteins

Here they are in their full glory—Peridinin-chlorophyll-protein complex (PerCP), Y-Phycoerythrin (YPE), R-Phycoerythrin (RPE), R-Phycocyanin (RPC), C-Phycocyanin (CPC) and Allophycocyanin (APC) (left to right). For the convenience of researchers, we have made some them available as kits for conjugation.

PhycoLink® Conjugation Kits

Product Code	Color	Emission
PJ31K	RPE	575 nm
PJ70K	BPE	575 nm
PJ25K	APC	660 nm
PJ40K	PerCP	675 nm
PJ90K	RT-665™	680 nm

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Slide 9 - PhycoLink® Conjugation Kits

These are the Product Codes for the various colors we offer. Our newest kit is PerCP, with which you're no doubt familiar. We're pleased that Becton, Dickinson and Company, Inc., who continues to hold the patent on this fluorochrome, recognized the increasing need for end-users to make their own conjugates of this useful fluor and agreed to license to us for use in conjugation kits for research use only. We also offer a tandem fluorochrome: RPE-Tandem-665™, which consists of RPE covalently coupled to the Oyster® 645 dye.

Slide 10 - PhycoLink® Conjugation Kits

Consider some of the features and benefits of our PhycoLink Conjugation Kits:

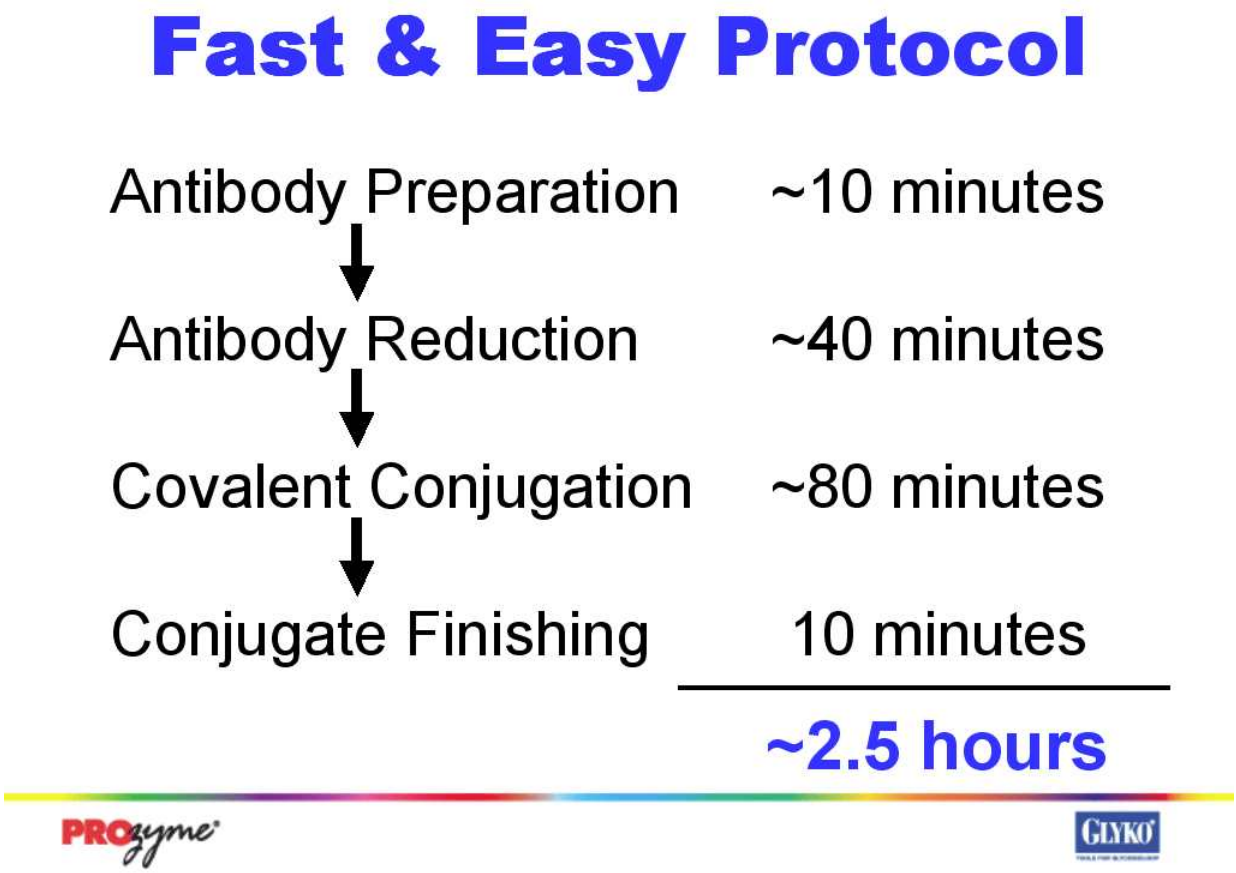
- First, they are **complete**. They contain everything you need to conjugate up to 1 mg of your antibody (or other target protein).
- They are **universal** in that they work with essentially any type of antibody molecule or other sulfhydryl-containing protein. The kits work exceptionally well with polyclonal and the majority of monoclonal antibodies. We've even had reports from customers who have successfully conjugated IgM's using our kits! And, we've successfully made $F(ab')_2$ conjugates ourselves.
- They're **fast**; the entire procedure takes less than 2 ½ hours to complete.
- The kits are extremely **easy to use**. We provide you with complete step-by-step protocols.
- Our kits utilize the PPP's, and as I've shown this should result in the **bright**est, most sensitive conjugates.
- The kits are configured to be **flexible** to allow different scale conjugations from a single kit. This is especially useful in cases where you wish to perform a trial

conjugation (*e.g.* 100 μg) and then scale-up.

- Lastly, they're **economical**. Making your own conjugates represents a cost-effective alternative to purchasing commercial conjugates, especially if you use a lot of the same conjugate; also, custom conjugations can cost thousands of dollars for similar quantities.

Slide 11 - Everything needed to conjugate up to 1 mg of antibody

PhycoLink kits contain everything you need to conjugate up to 1 mg of antibody (2 mg in the case of the PerCP kit): most importantly, the activated phycopigment protein; and all the other necessary reagents; two different gel filtration methods (desalting columns and spin columns) to enable different scale conjugations; and pre-made exchange and storage buffers, plus the recipes for making more.

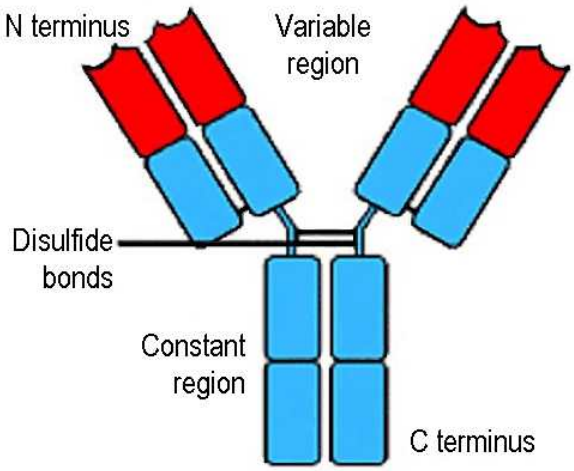


Slide 12 - Fast & Easy Protocol

The procedure takes less than 2 ½ hours from start to finish and consists of four easy steps: antibody preparation, antibody reduction, covalent conjugation and conjugate finishing.

Antibody Preparation & Reduction

- Start with a purified antibody solution
- Reduce antibody to expose free sulfhydryls
- Remove excess reducing agent



C. A. Janeway Jr. in *Immunobiology*. P. Austin and E. Lawrence eds. Garland, Publishing, New York, NY (2001).

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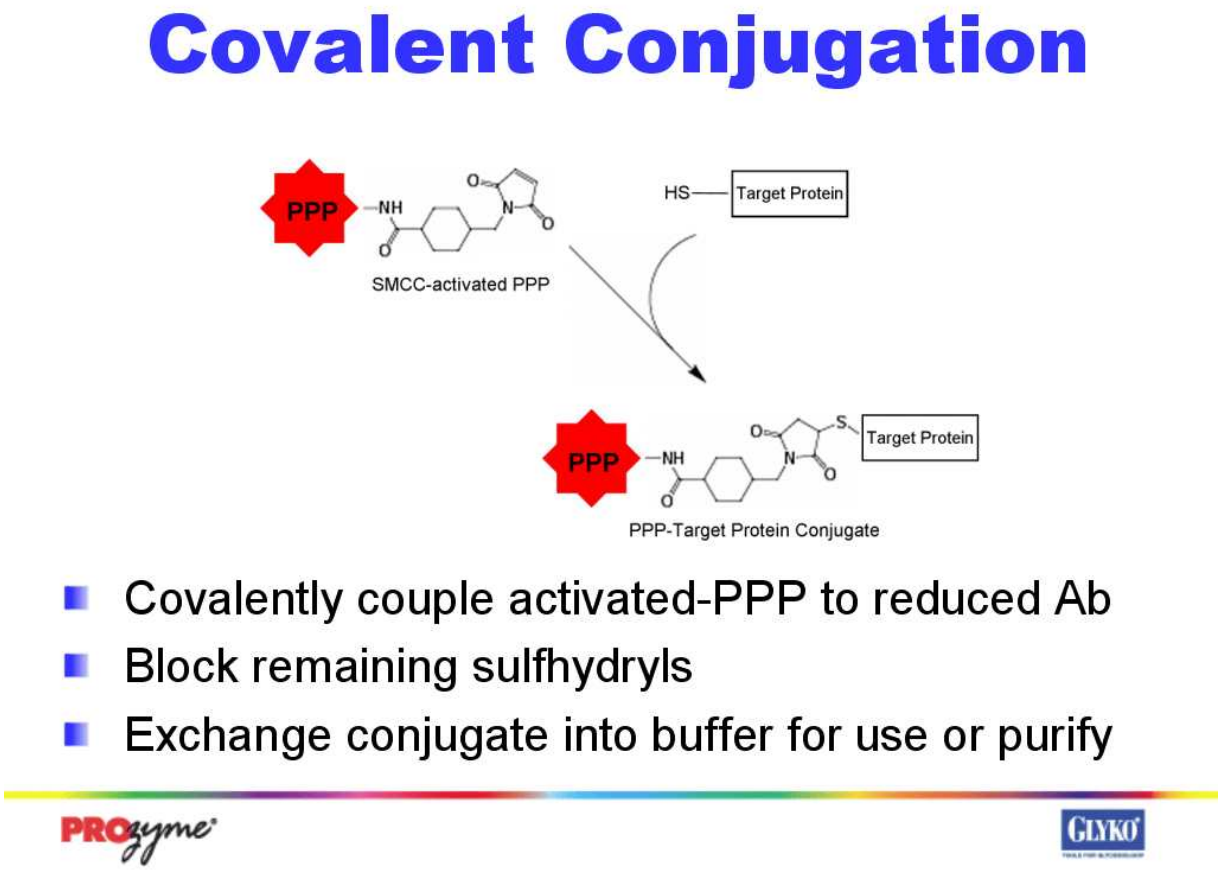
Slide 13 - Antibody Preparation & Reduction

It's important to start out with a purified antibody solution, preferably at 1-10 mg/ml for best results. Essentially any neutral buffer is compatible (*e.g.* phosphate, Tris, MES), but the solution should be free of BSA or other sulfhydryl-containing proteins. If you're planning to conjugate an antibody you've purchased from an outside source, always request a copy of the Certificate of Analysis from the vendor which specifies the formulation, including any carrier proteins. Additives such as sodium azide or glycerol, up to a certain amount, shouldn't interfere, but we've got a whole series of Frequently Asked Questions (FAQ's) on our website which discuss buffer considerations, concentrations and other common questions:

http://www.prozyme.com/faqs/faqs.html#phycolink

Start by reducing your purified antibody to expose free sulfhydryls. Our standard protocol is preferential for the hinge disulfides. Again, this is important in that the conjugation process won't interfere with or diminish the antibody's antigen binding capacity.

Before proceeding to the covalent conjugation step it's important to remove the excess reducing agent. This is accomplished with either the desalting or spin columns provided.



Slide 14 - Covalent Conjugation

Once you've reduced and desalted your antibody, covalently couple it to the activated PPP. The activated PPP provided in the kit is a fully tested product in and of itself and is available for purchase separately.

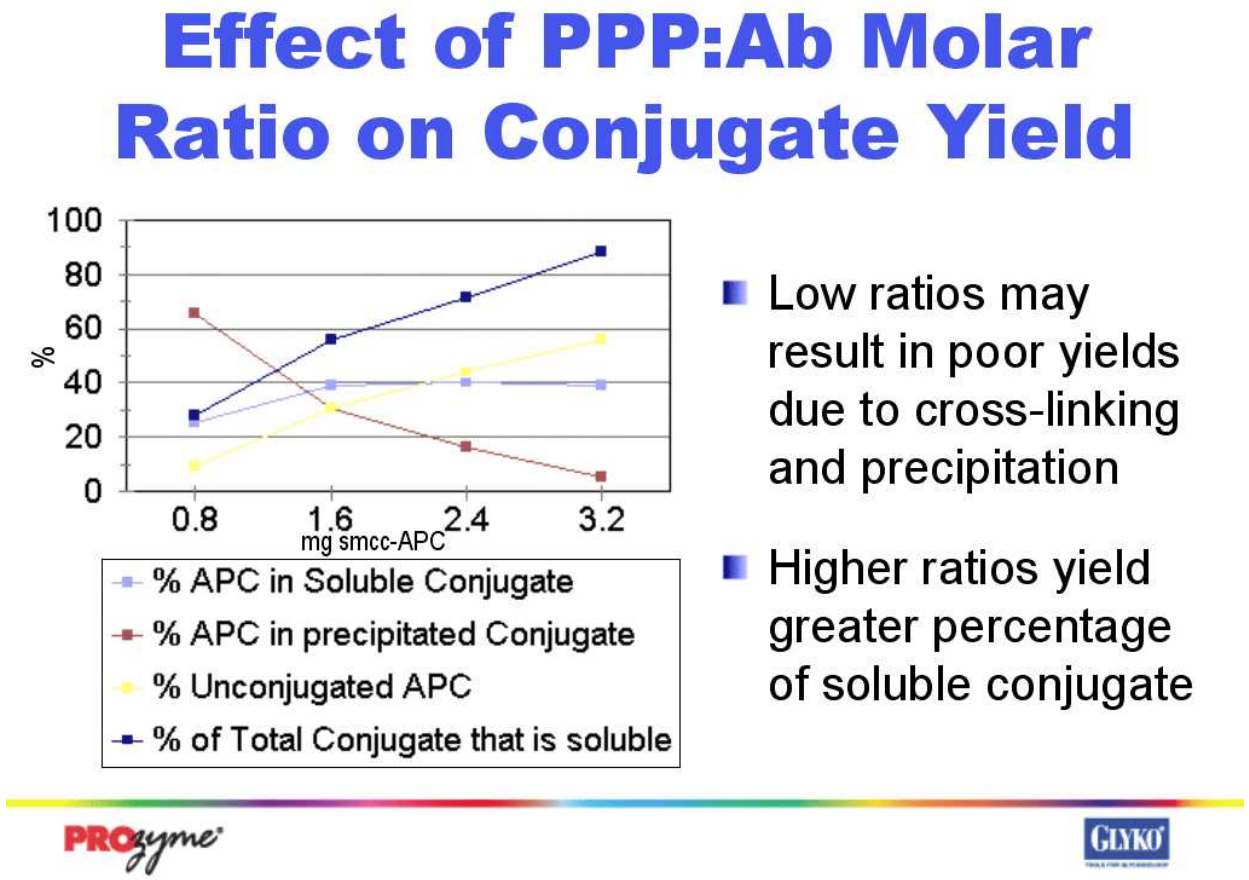
The conjugation conditions have been optimized to ensure the reaction works as reliably as possible with the majority of antibodies. A molar excess of activated PPP is added to drive the reaction so that practically every antibody molecule is tagged.

This slide shows an oversimplified diagram of what is actually occurring. In reality, there are likely to be several lysine residues modified per PPP molecule (in other words, several SMCCs per PPP). Also, each antibody contains more than one free sulfhydryl residue, although usually only 1-2 activated PPPs react per antibody due to steric hindrance. This has the potential to produce antibody-PPP molecules of varying composition.

Although reagent manufacturers often report the molar ratio of antibody-to-fluor in their conjugates as 1:1, this can be misleading and should not be taken to mean that every single antibody-PPP molecule is composed of only one antibody plus one PPP. The conjugate is usually a heterogeneous population of different antibody-fluorochrome molecules, each characterized by their own antibody-to-PPP ratios (*e.g.* 1:1, 2:2, 3:3; or even 2:3 or 3:2). The aggregate ratio of the overall population may average out to 1:1, or

to a higher or lower ratio.

Following covalent conjugation, any remaining free sulfhydryl groups are covalently blocked by treatment with NEM, and the conjugate may either be exchanged into Storage Buffer for immediate use or purified using one of our Purification Kits.

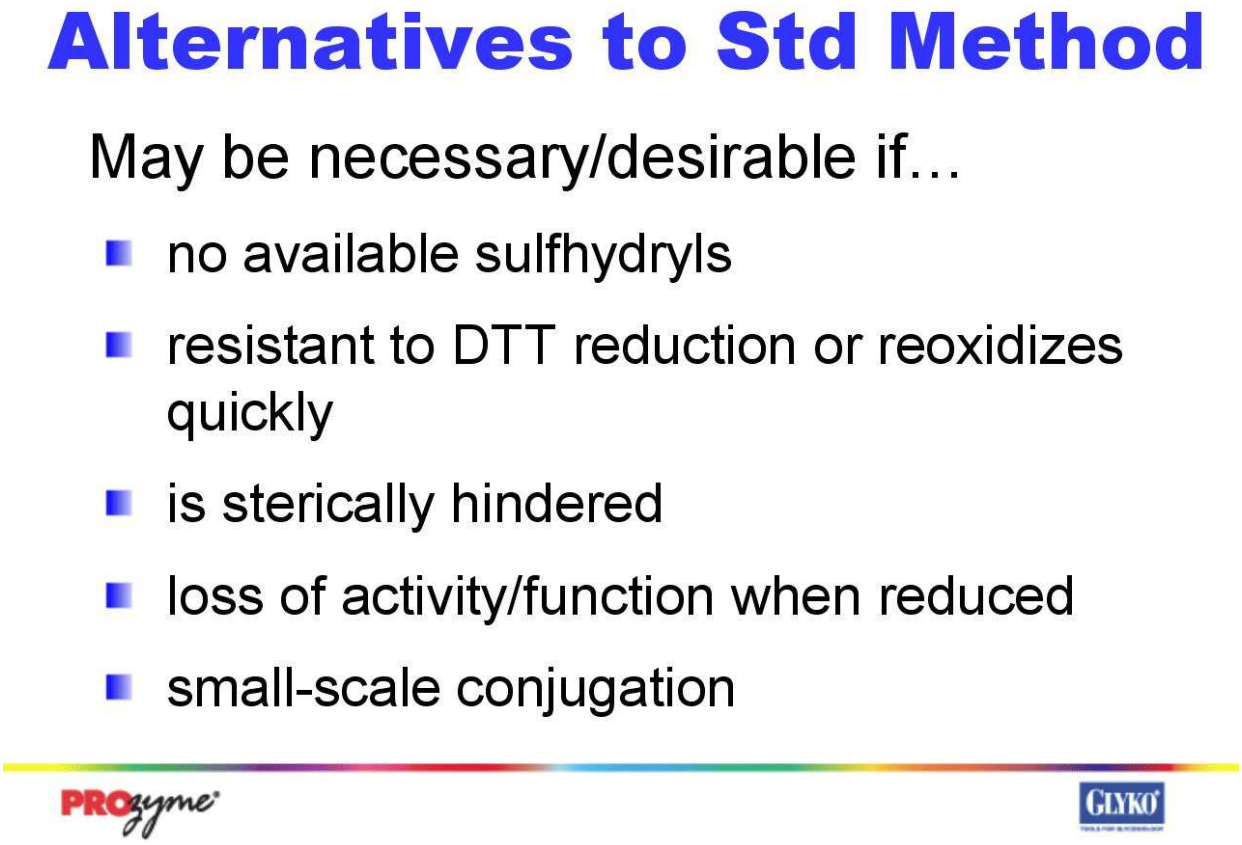


Slide 15 - Effect of PPP:Ab Molar Ratio on Conjugate Yield

This slide summarizes the effect of varying the molar ratio of PPP to antibody on conjugate yield. For a full discussion, please refer to the FAQ on our website:

http://www.prozyme.com/faqs/pjpamtfaq.html

In this experiment, 1 mg of purified goat IgG was conjugated with various amounts of activated APC (x-axis). The reddish-brown curve shows the percentage of APC in precipitated conjugate while the dark blue line shows the percentage of soluble conjugate. While low molar ratios of APC to Ab give high incorporation of APC into conjugate, as you can see (reddish-brown line) they can result in poor yields due to excessive cross-linking and precipitation; whereas high molar ratios of APC to Ab result in a greater percentage of soluble conjugate (dark blue line). We've selected ratios for our kits that should produce high yields with very little precipitation if you follow our standard protocol. *NOTE: there will be excess PPP not incorporated into the conjugate as a result.* Further, every PPP has its own optimal molar ratio; what's shown here is specific to APC.



Slide 16 - Alternatives to the Standard Method

In some cases, it may be necessary or desirable to use an alternative conjugation method for a number of reasons: your protein doesn't contain any endogenous sulfhydryls; is resistant to DTT reduction; reoxidizes quickly; is sterically hindered or loses activity/function when reduced; or to minimize losses or dilution when performing small-scale conjugations.

Alternatives to Std Method

Method	Protein contains sulfhydryls	Protein does not contain sulfhydryls
SPDP-TCEP	yes	yes
Iminothiolane	yes	yes
Direct TCEP **(under development)**	yes	no

See our **TechNote TNPJ300**

Slide 17 - Alternatives to the Standard Method (continued)

There are three different methods for conjugating your protein using the activated PPP provided in our conjugation kits when an alternative to our standard protocol is needed. The choice of which method to use will depend on several factors: whether your protein contains available sulfhydryls; whether or not you wish you reduce your protein; and the scale of the conjugation reaction.

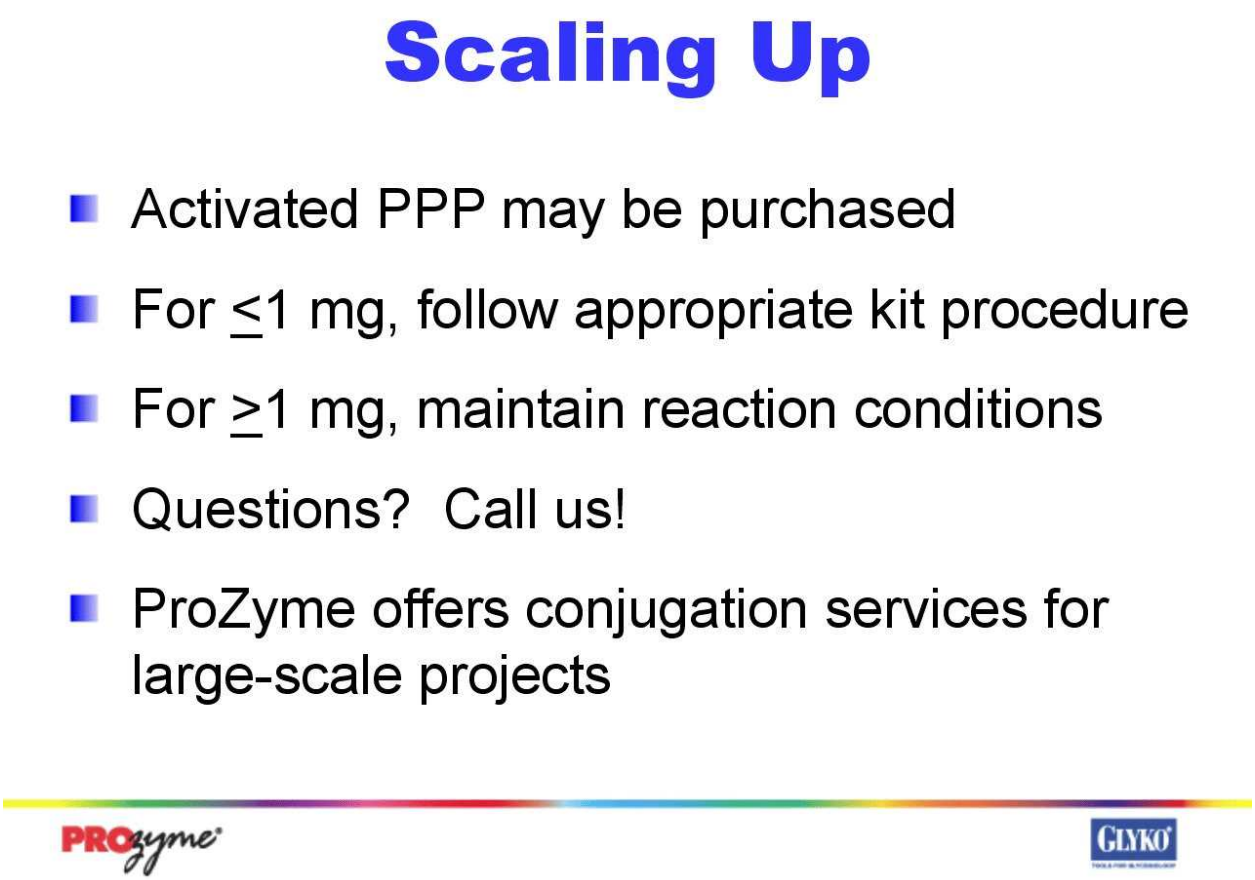
The first two methods (Iminothiolane and SPDP-TCEP) work with both sulfyhdryl- and non-sulfhydryl-containing proteins. The Iminothiolane method has the advantage that it avoids a reduction step, but has the disadvantage that it works through amine modification which, in the case of antibodies, has the potential to interfere with the antigen binding site. This method is also subject to the same issue of loss and dilution as our standard method when performing small-scale conjugations. The SPDP-TCEP method has an advantage in this regard in that it doesn't require desalting but, like Iminothiolane, has the potential to result in interference with the antigen binding site.

The third method (Direct TCEP), is a new one which we hope to have incorporated into our kits within the next couple of months. This method may be suitable when performing small-scale (*e.g.* ≤0.25 mg) conjugations of sulfyhdyryl-containing proteins. As a non-thiol containing reducing agent, TCEP doesn't require desalting and thus avoids the losses and/or dilution associated with DTT removal. However, it should be noted that TCEP is

purported to be a more potent reducing agent than DTT, which may affect the characteristics of a given antibody.

For more details concerning alternative conjugation methods, see our TechNote TNPJ300:

http://www.prozyme.com/pdf/tnpj300.pdf



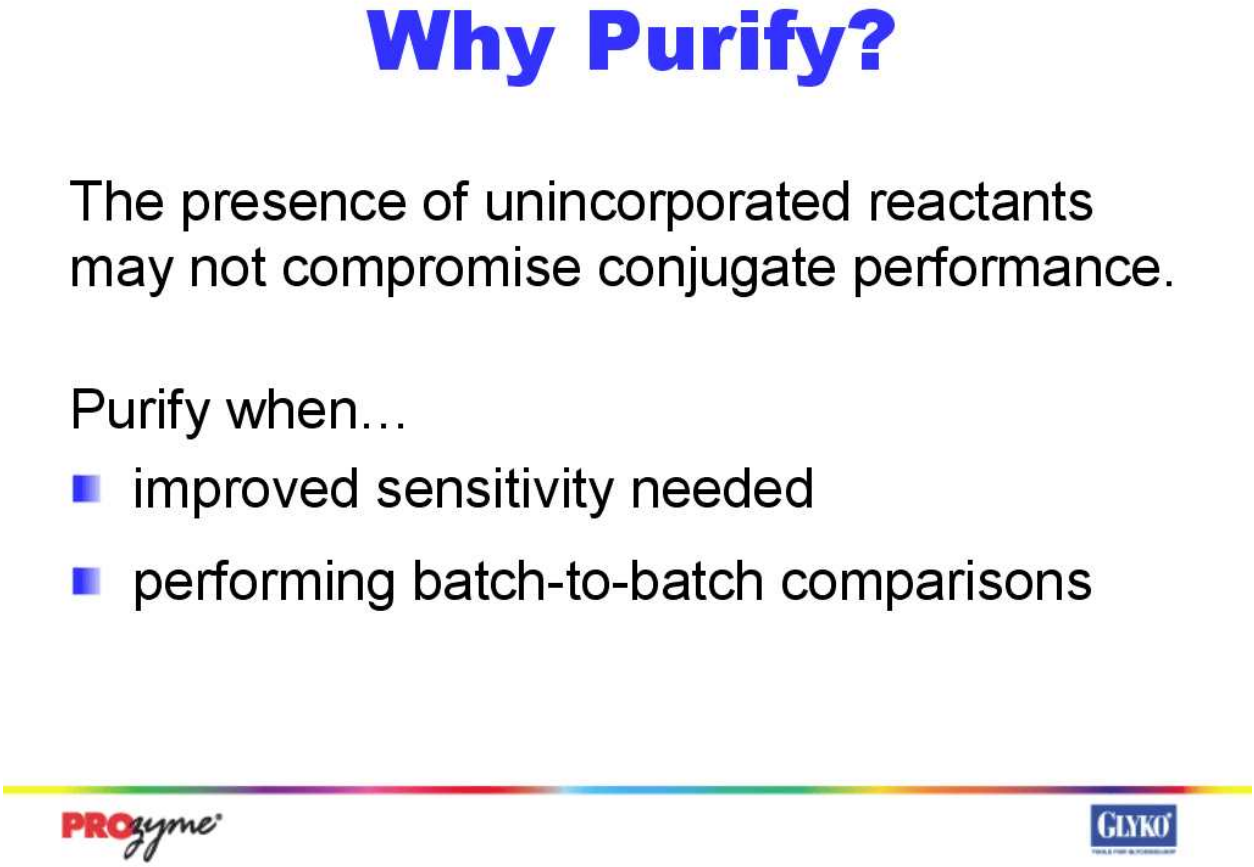
Slide 18 - Scaling Up

Let's assume you've successfully made a conjugate (*e.g.* 0.1 mg), tested it in your application and now wish to scale-up.

For scale-ups within the capacity of our kits, we recommend following the procedure appropriate for the amount of antibody: 0.1 mg to 0.25 mg conjugations are best performed using the Spin Column procedure; 0.5 mg to 1 mg conjugations are best performed using the Desalting Column procedure; 0.25 mg to 0.5 mg conjugations may be performed using either procedure, depending on antibody concentration.

Scale-ups beyond the capacity of our kits may be performed by purchasing additional activated PPP. While a full discussion of the parameters relevant to scale-up is beyond the scope of this presentation, by maintaining antibody concentration and molar ratios, we've been able to demonstrate an essentially linear scale-up from 0.5 mg to ~2 mg to 10 mg and beyond. We invite you to give us a call if you are contemplating a scale-up, as individual antibodies have unique considerations and actual results may vary.

In case you'd prefer to spend your time on other exciting aspects of your research, ProZyme offers affordable custom conjugation services for large-scale projects.

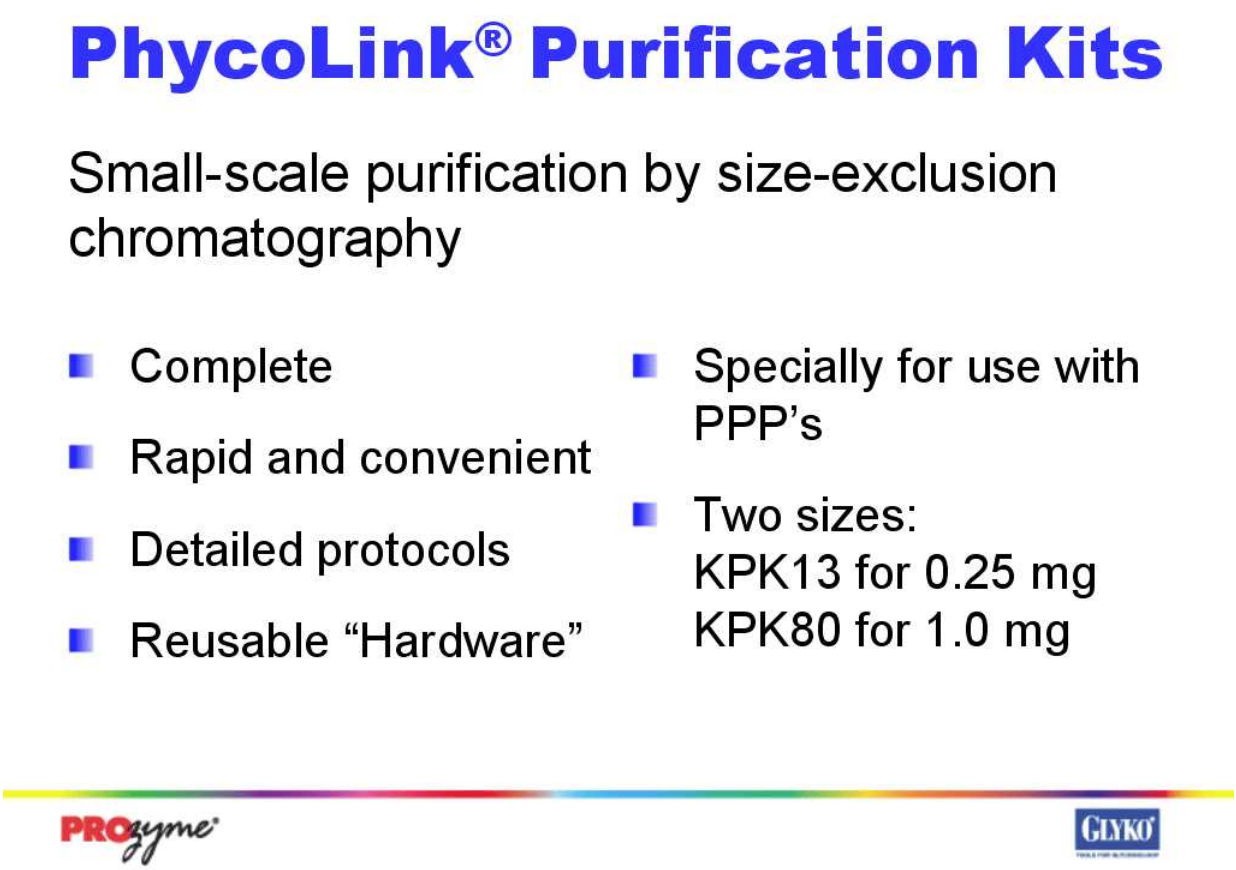


Slide 19 - Why Purify?

In many cases, it's possible to use your conjugates "as is", with some unincorporated PPP. This often doesn't pose a problem, especially when staining cell surface antigens, as unbound PPP is washed away in the process.

However, depending on the application, purification of unincorporated activated PPP may be desirable in order to achieve higher sensitivity through a reduction of non-specific binding. Flow cytometric staining of intracellular antigens is an example of an application which may benefit by the use of purified conjugates. It's also necessary to purify your conjugate if you intend to perform lot-to-lot comparisons as the unincorporated activated PPP will interfere in the evaluation.

The disadvantages of purification should be taken into consideration when deciding whether or not to purify as losses can exceed 50% when processing small quantities. In general, our recommendation is that you not purify unless absolutely necessary. Sometimes only a small portion needs to be purified for evaluation.



Slide 20 - PhycoLink® Purification Kits

If you've decided to purify your conjugate, we offer purification kits to allow rapid and convenient purification by means of size exclusion chromatography. Our kits contain everything necessary to remove unincorporated reactants from your conjugate and include detailed protocols that have been thoroughly tested for trouble-free purification. When you purchase a kit, you also receive a CD-ROM with photographs to guide you through the various steps.

Importantly, our purification kits are specially formulated for use with PPP conjugates. The PPP's are very large molecules (*e.g.* RPE is ~240 kDa; APC is ~104 kDa). So, the challenge is to purify away one large molecule (the activated PPP) from another large molecule (your conjugate). This isn't effectively done with any ordinary matrix. We've identified the best matrix for this purpose and have selected one that achieves optimal separation while minimizing non-specific binding of PPP's, known to be a problem with certain matrices.

NOTE: Affinity purification is not recommended for PPP conjugates as the harsh elution conditions diminish fluorescent properties.

Presently, our purification kits are available in two sizes:

KPK13: 13-ml column for purification of 0.25 mg conjugates
KPK80: 80-ml column for purification of 1.0 mg conjugates

but other column sizes are generally available that work with the kit connectors.

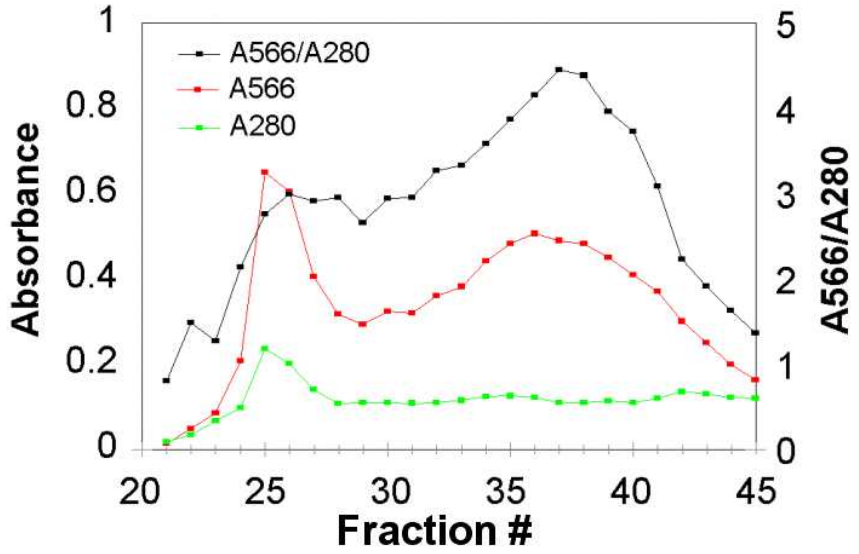
Better resolution can be achieved with the 80-ml column than the 13-ml column due to its greater length, and hence, resolving capability. However, it is possible to obtain significantly purified conjugate with either column by selecting only early fractions.

A single purification kit can be used many times; the hardware is reusable and the matrix is stable when stored in ethanol at 4°C. Some investigators prefer to designate a different matrix batch for each conjugate. Matrix refills may be purchased from ProZyme for only a fraction of the cost of the original kit.

The set-up is based on a Luer-Lock™ system, so it is possible to substitute a different column to accommodate the amount of conjugate. For example, one of our customers, who happens to be attending this meeting, Dr. Jian Ling from the Southwest Medical Research Center in San Antonio, Texas, wished to purify a 50-μg conjugate he had made using one of our conjugation kits. We were concerned that, due to the small volume, the losses with our 13-ml column would be too great. Dr. Ling independently acquired a 4-ml column, followed our setup procedures (using our matrix) and was able to achieve adequate separation of his conjugate from the unreacted components in order to perform an intracellular flow experiment requiring high sensitivity.

(Thank you Dr. Ling for allowing us to use your name as an example.)

Mouse IgG-RPE:
80-ml Column Profile



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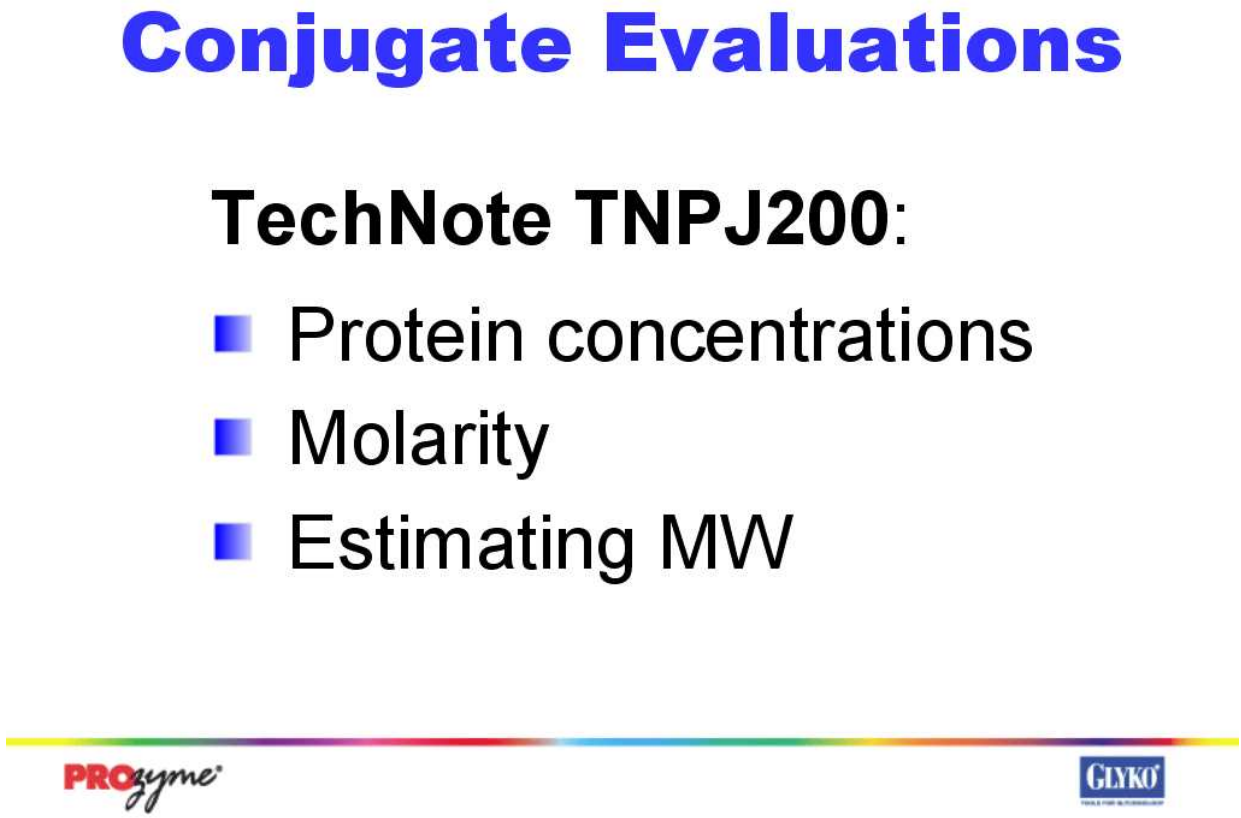
Slide 21 - Mouse IgG-RPE: 80-ml Column Profile

This is an example of a typical column profile obtained with our 80-ml column. We're looking at fractions of a 1-mg mouse IgG-RPE. As you can see, the majority of the conjugate elutes as a leading peak, with smaller conjugate complexes running between the leading peak and the broad peak of unincorporated RPE. Very little unincorporated antibody (green line) is present due to the optimized protocols.

In choosing which fractions to pool, there's a trade-off between purity and yield. Clearly, the early fractions will be the most pure, but if your application can tolerate it, less pure fractions may be included in order to increase yield.



Slide 22 - Conjugate Evaluations

Please refer to TechNote TNPJ200 for a complete set of tools for evaluating your purified conjugate:

http://www.prozyme.com/pdf/tnpj200.pdf

This TechNote contains techniques for determining protein concentration, molarity of individual components, molecular weight and conjugate composition. For those of you who may be a little rusty in terms of your concentration calculations, the TechNote includes a worksheet with all the formulas necessary to convert absorbance measurements into concentration data; all you have to do is fill in the blanks.

These characterization techniques may be applied both to conjugates made using our kits as well as those purchased from commercial sources. Although conjugate manufacturers would like us to believe in the simplified case that conjugates are 1:1, this probably isn't so often the case. Therefore, you may be well served to characterize the conjugates you're purchasing to determine the fluorochrome-to-antibody ratio, particularly if the intended use is for quantitative analysis.

Technical Support

- All protocols, FAQs and TechNotes are available on our website:

  http://www.prozyme.com/phycolink/pj-kits.html

- Contact us if things go wrong

PROzyme GLYKO

Slide 23 - Technical Support

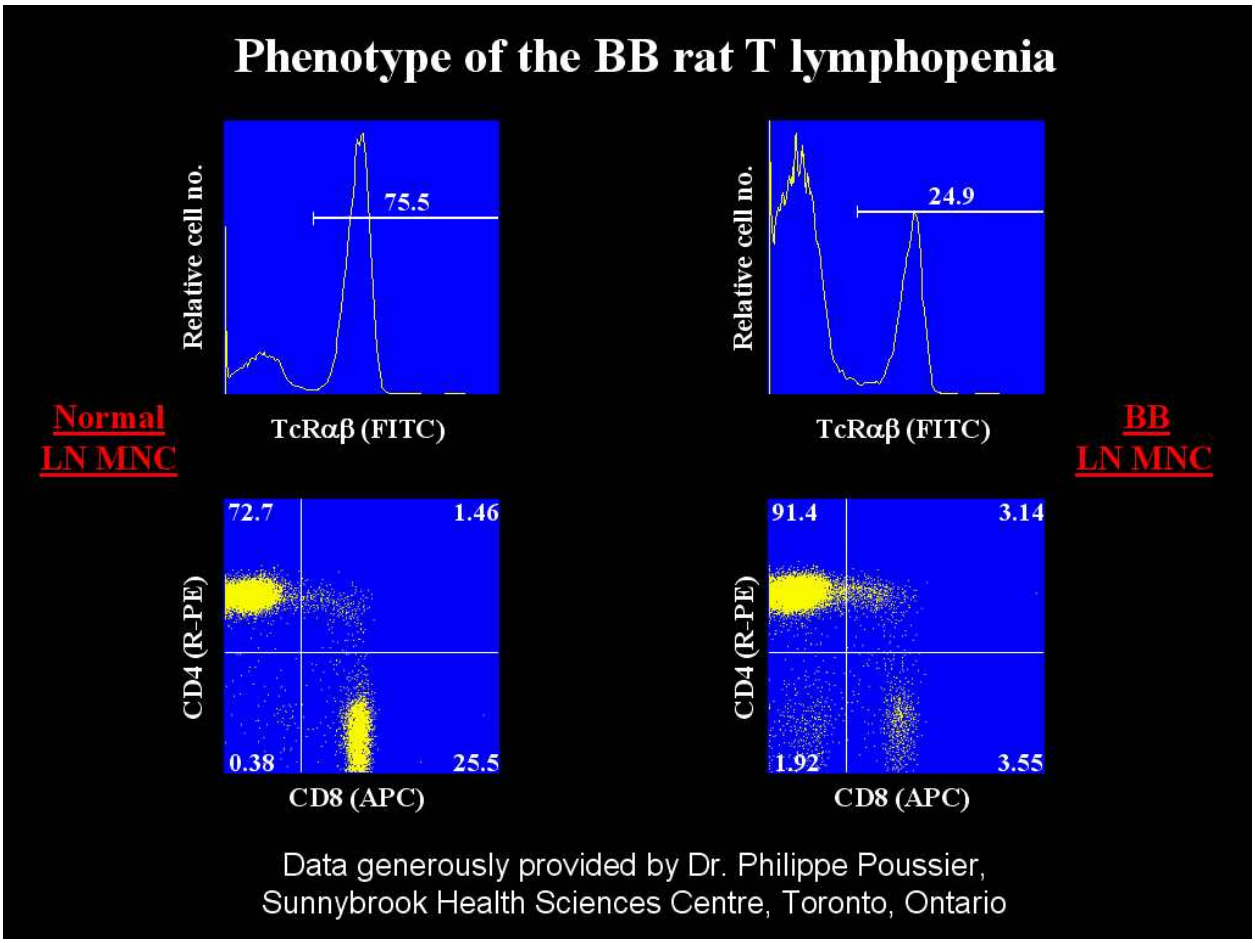
All of the protocols, FAQs and TechNotes referenced, are available on our website:

http://www.prozyme.com/phycolink/pj-kits.html

Our kits work well with the majority of antibodies, and customers rarely have problems. However, I want to emphasize that we're here to help at any step along the way. We appreciate that your time is valuable, so the first thing we ask you to do, if you don't get the expected results, is to complete the troubleshooting form in the FAQ section on our website (and submit it to us by email or fax):

http://www.prozyme.com/faqs/pjperffaq.html

That way, we'll have all the information concerning your experiment and be as prepared as possible to provide a solution to the problem.



Slide 24 - Phenotype of the BB Rat T Lymphopenia

Now, I'd like to turn to some data supplied by our customers. This first example is work done in the laboratory of Dr. Phillippe Poussier at Sunnybrook Health Sciences Centre in Toronto, Ontario.

Shown are lymph node mononuclear cells from the diabetes-prone BB rat (right panels) versus control animals (left panels), stained with either anti-TCR α/β FITC (top row) or CD4-RPE and CD8-APC (bottom row). Note, the Gimap5 mutation in the BB rat results in severe T lymphopenia, including distortion of the normal CD4:CD8 ratio.

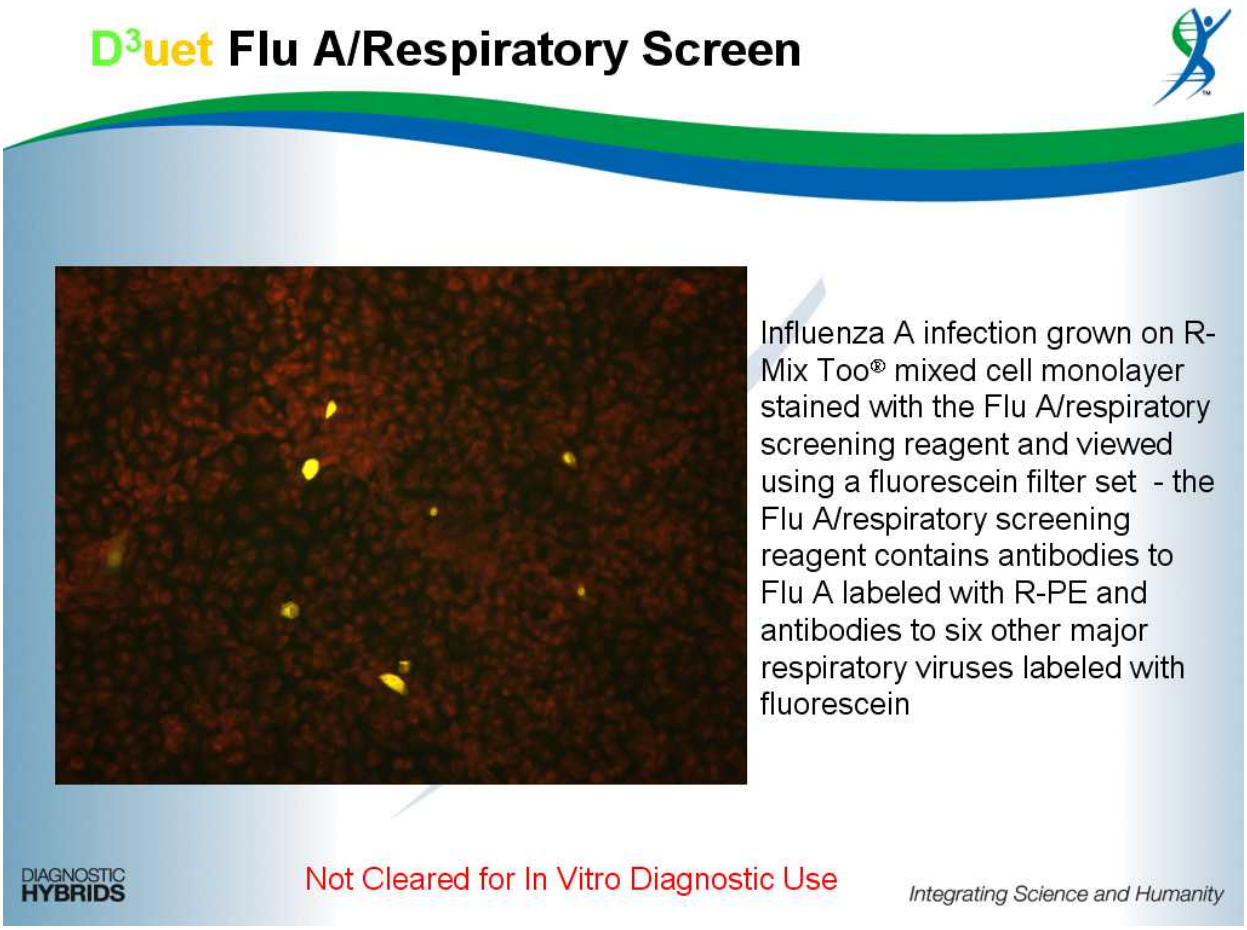
The antibodies used in this experiment were made in-house from hybridomas (all mouse IgG1-kappa) purchased from the European Tissue Culture Collection; and the CD4-RPE and CD8-APC conjugates were made using PhycoLink Conjugation Kits. Dr. Poussier's lab typically performs 1-mg conjugations, but has done smaller-scale conjugations with acceptable results. The conjugates are used without purification, yet as you can see, bright staining of the respective populations is achieved, with very little background.

According to Dr. Poussier, his lab makes their own conjugates, as opposed to purchasing commercial conjugates, because "it is cheaper in the long run especially if several investigators who use the same reagents share the costs."

Slides 25 through 31

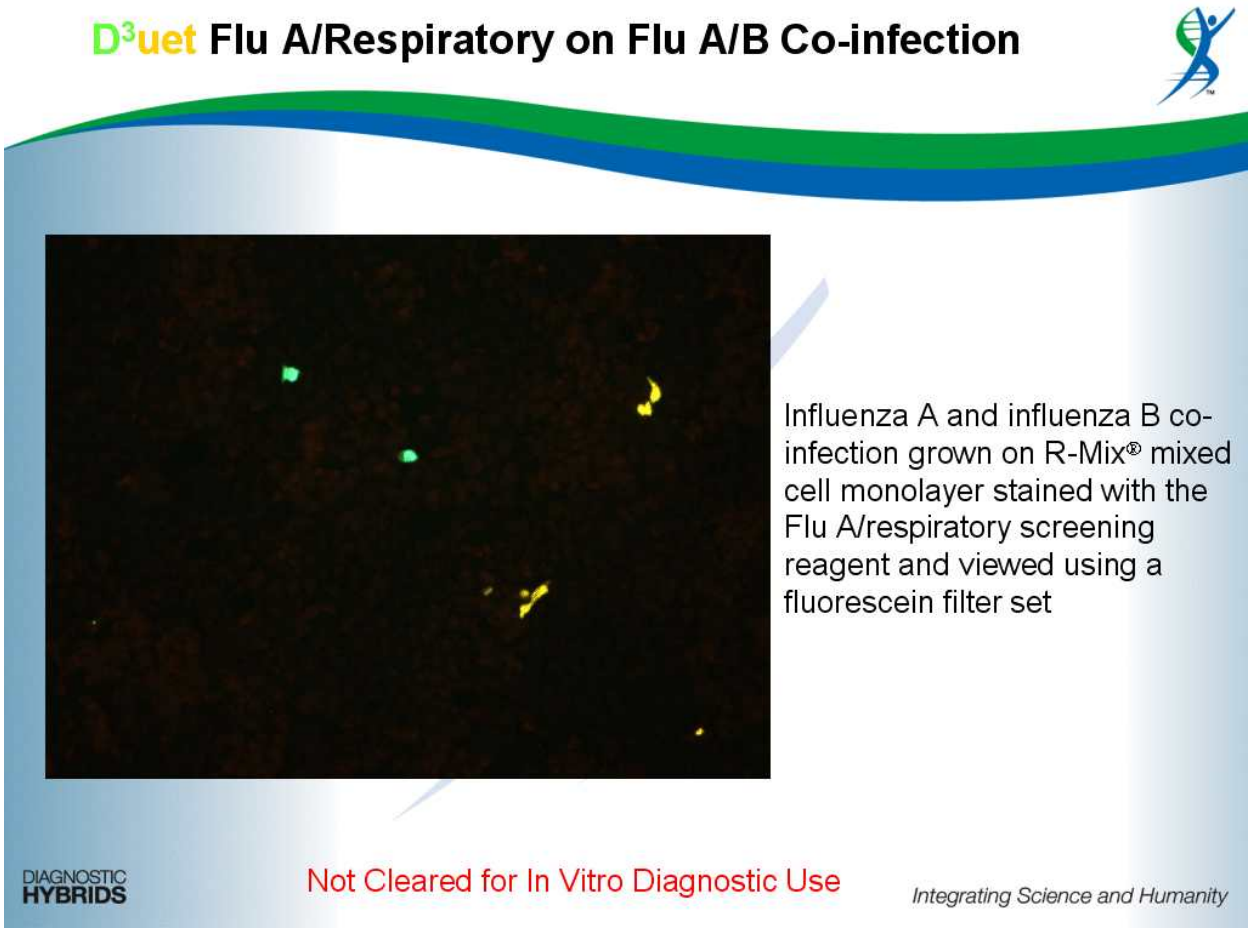
The next series of slides was kindly provided by Diagnostic Hybrids, based in Athens, Ohio, who are developing a clinical diagnostic assay for the rapid detection of viral infection based on a conventional fluorescence microscopy platform. Their assay makes use of highly-specific anti-viral monoclonal antibodies conjugated to RPE, which when viewed through a fluorescein filter set appears yellow. They are planning to submit a 510(k) application to the FDA in order to obtain clearance for these assays as *in vitro* diagnostic (IVD) Reagents and are currently looking for partner sites who would be interested in performing clinical testing.

The PPP's traditionally have been excluded from use as tags for fluorescence microscopy due to concerns about photobleaching. Diagnostic Hybrids has observed absolutely no evidence of photobleaching of their RPE conjugates, even under the continuous illumination conditions inherent in this platform.



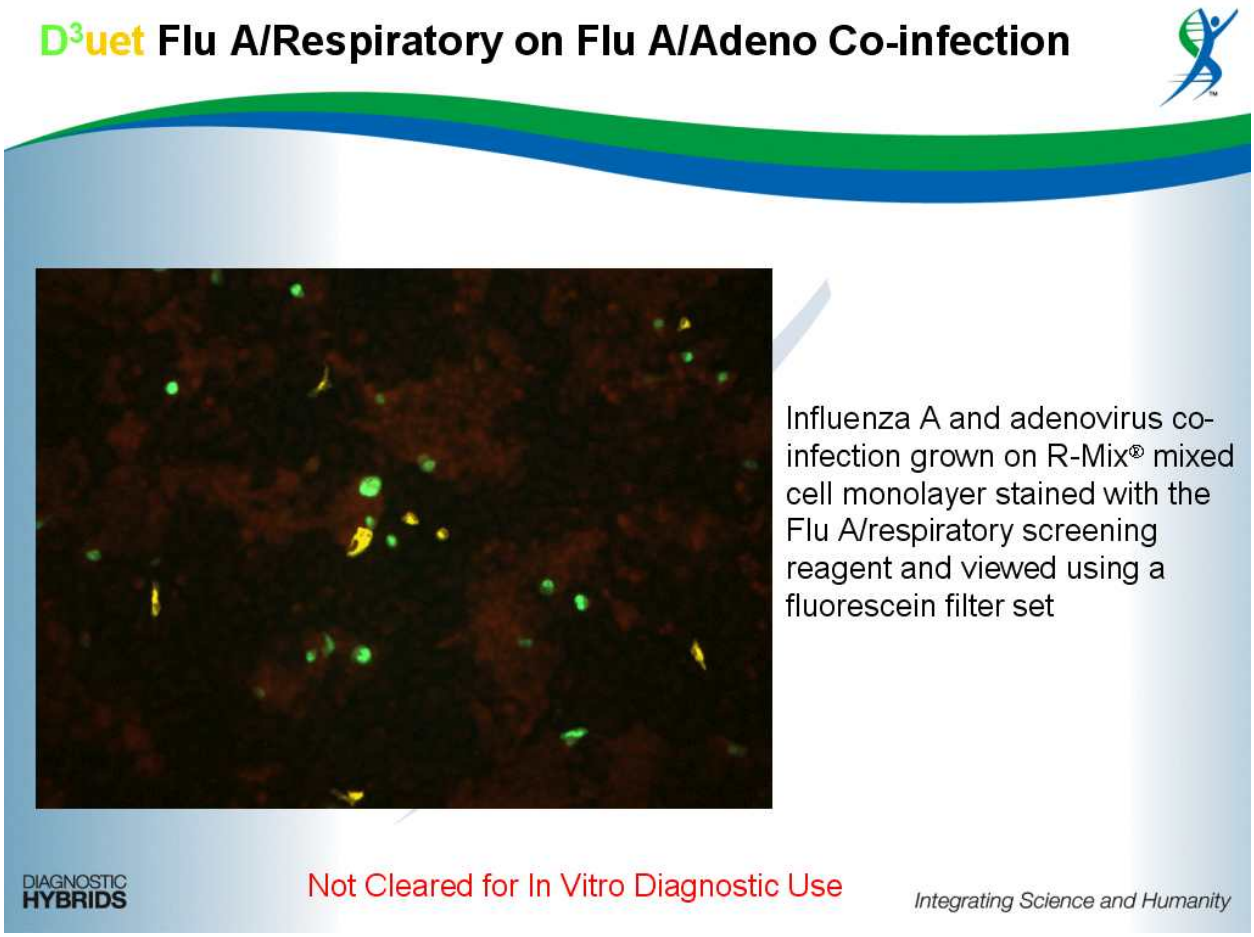
Slide 25 - D³uet Flu A/Respiratory Screen

Influenza A infection grown on R-Mix Too® mixed cell monolayer stained with the Flu A/respiratory screening reagent and viewed using a fluorescein filter set. The Flu A/respiratory screening reagent contains antibodies to Flu A labeled with RPE and antibodies to six other major respiratory viruses labeled with fluorescein.



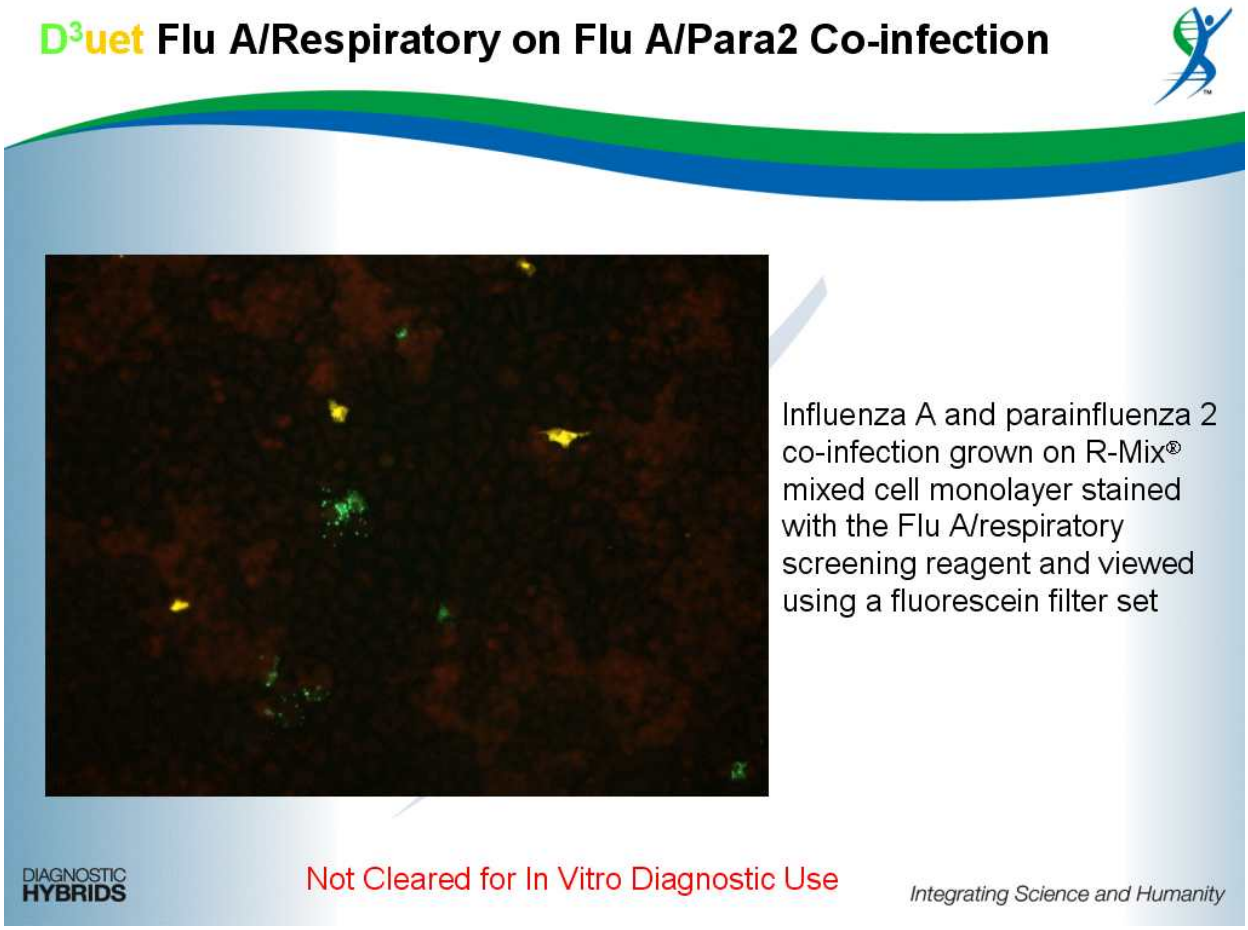
Slide 26 - D³uet Flu A/Respiratory on Flu A/B Co-infection

Influenza A and influenza B co-infection grown on R-Mix® mixed cell monolayer stained with the Flu A/respiratory screening reagent and viewed using a fluorescein filter set.



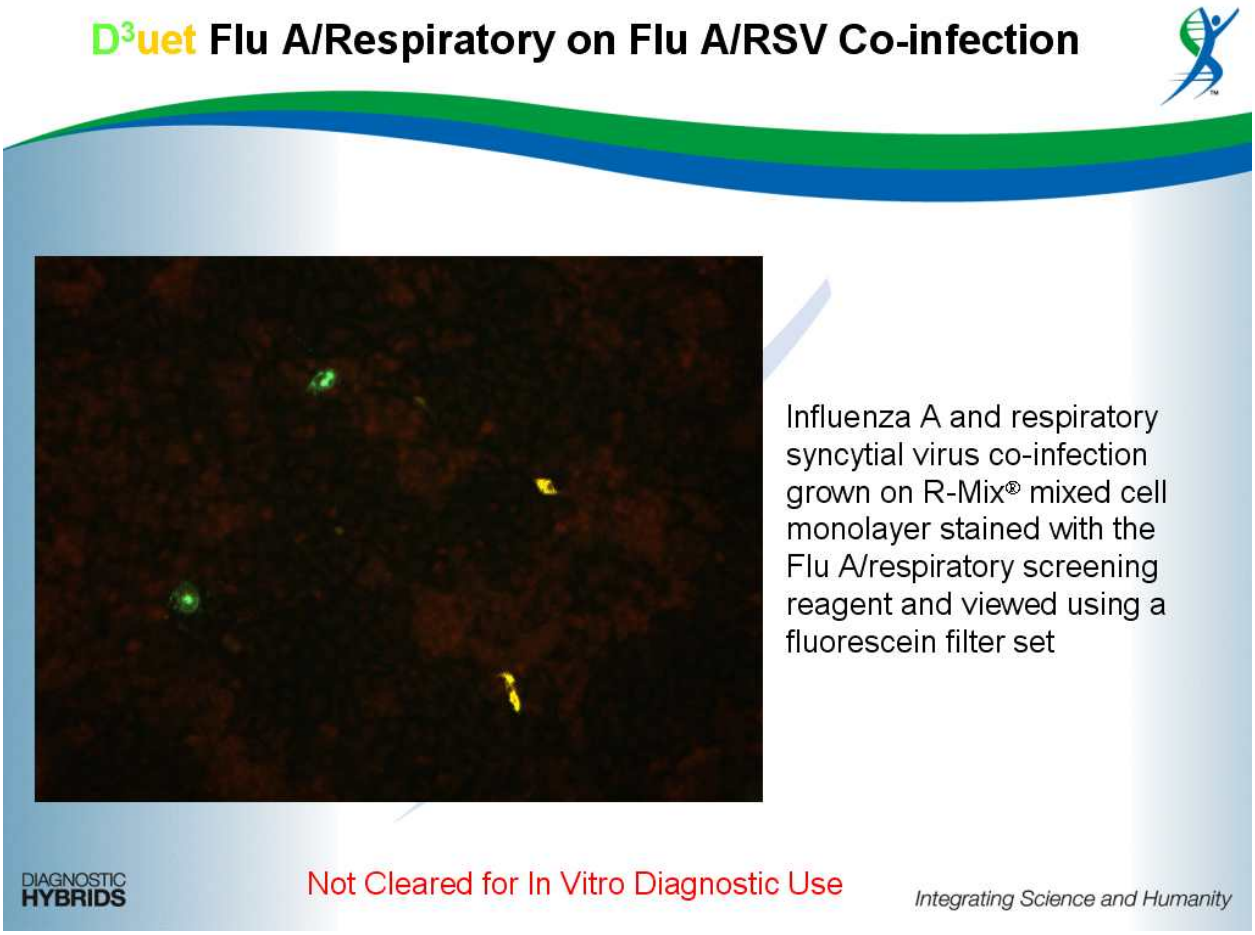
Slide 27 - D³uet Flu A/Respiratory on Flu A/Adeno Co-infection

Influenza A and adenovirus co-infection grown on R-Mix® mixed cell monolayer stained with the Flu A/respiratory screening reagent and viewed using a fluorescein filter set.



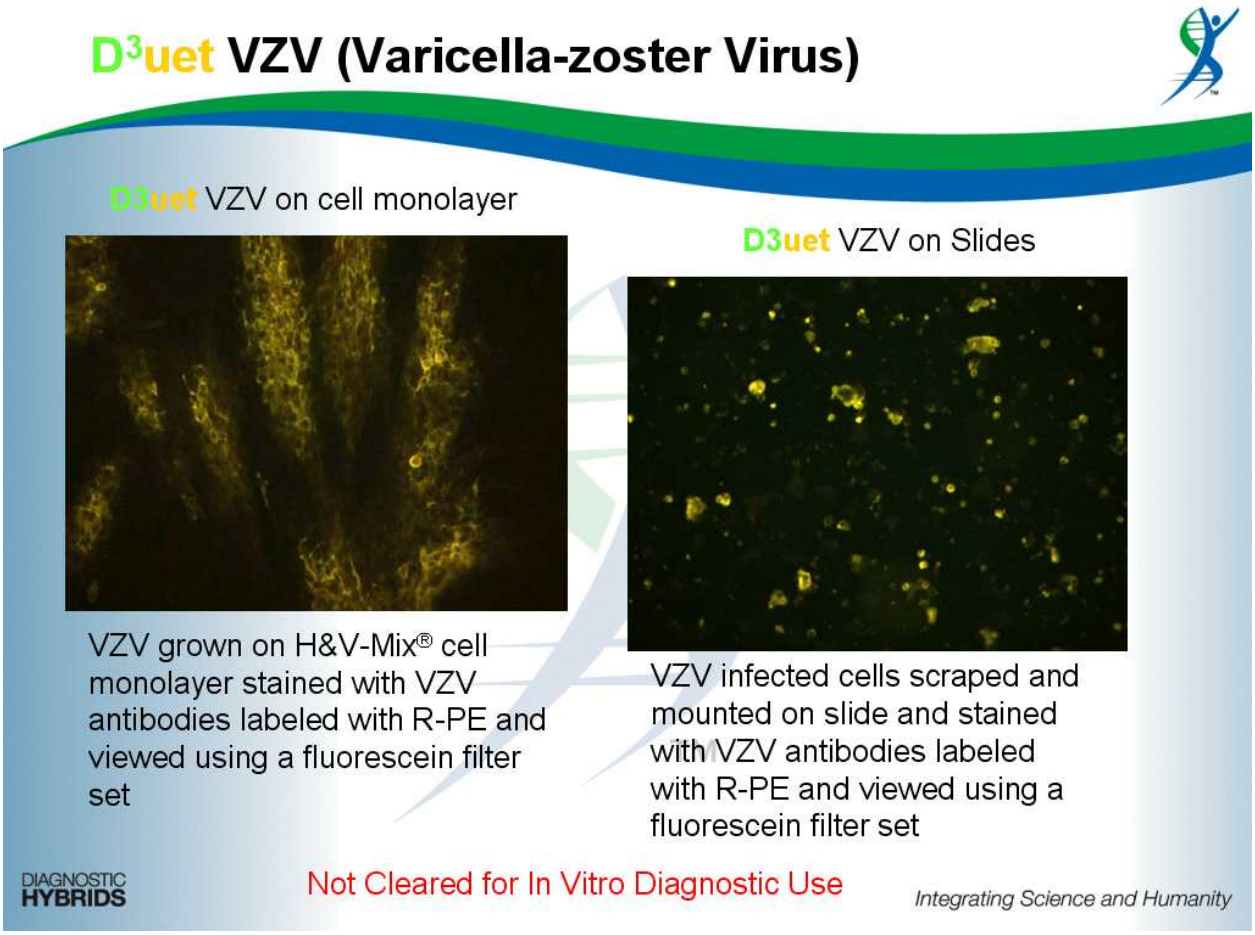
Slide 28 - D³uet Flu A/Respiratory on Flu A/Para2 Co-infection

Influenza A and parainfluenza 2 co-infection grown on R-Mix® mixed cell monolayer stained with the Flu A/respiratory screening reagent and viewed using a fluorescein filter set.



Slide 29 - D³uet Flu A/Respiratory on Flu A/RSV Co-infection

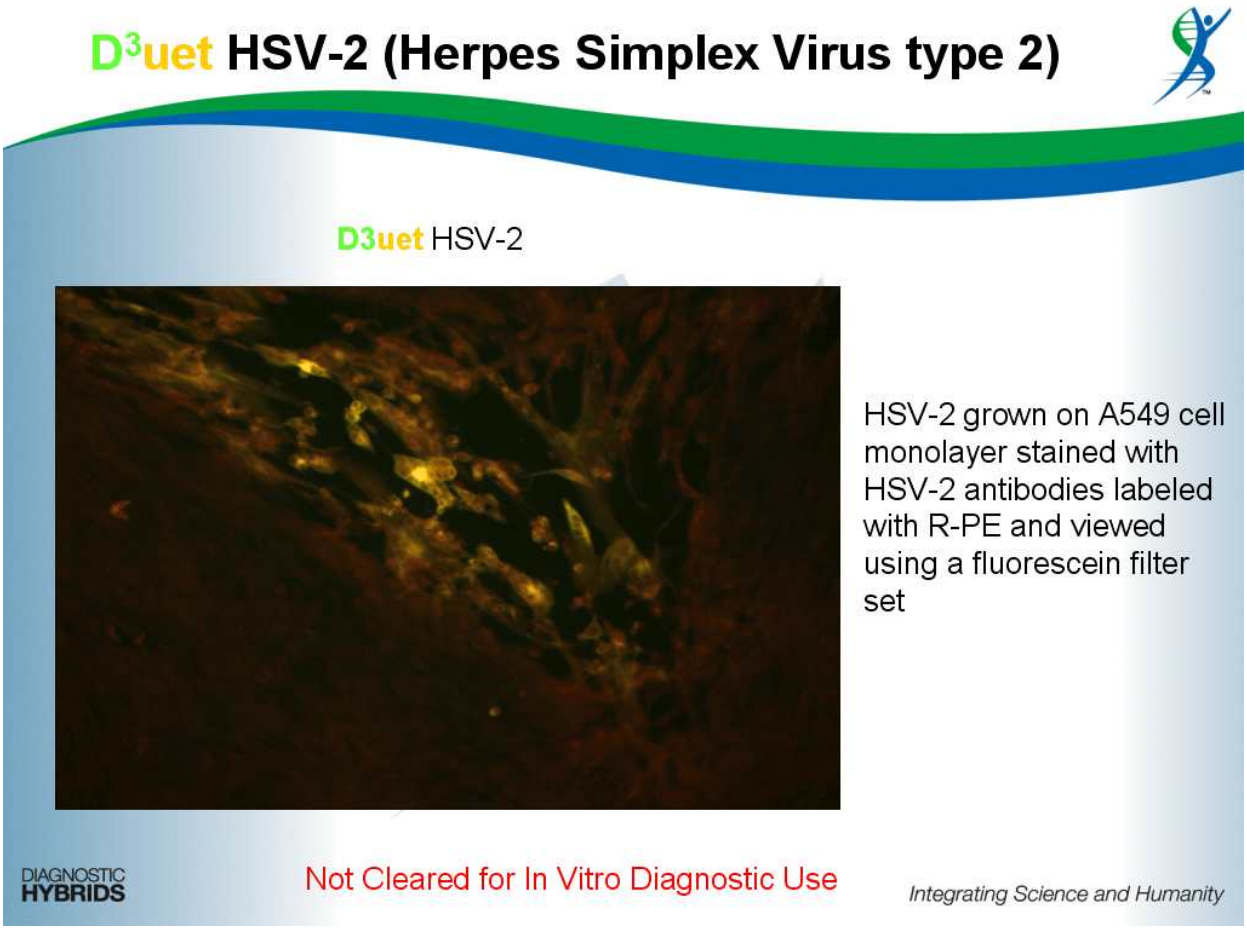
Influenza A and respiratory syncytial virus co-infection grown on R-Mix® mixed cell monolayer stained with the Flu A/respiratory screening reagent and viewed using a fluorescein filter set.



Slide 30 - D^{3}uet VZV (Varicella-zoster Virus)

Left-hand panel: VZV grown on H&V-Mix® cell monolayer stained with VZV antibodies labeled with RPE and viewed using a fluorescein filter set.

Right-hand panel: VZV infected cells scraped and mounted on slide and stained with VZV antibodies labeled with RPE and viewed using a fluorescein filter set.



Slide 31 - D³uet HSV-2 (Herpes Simplex Virus type 2)

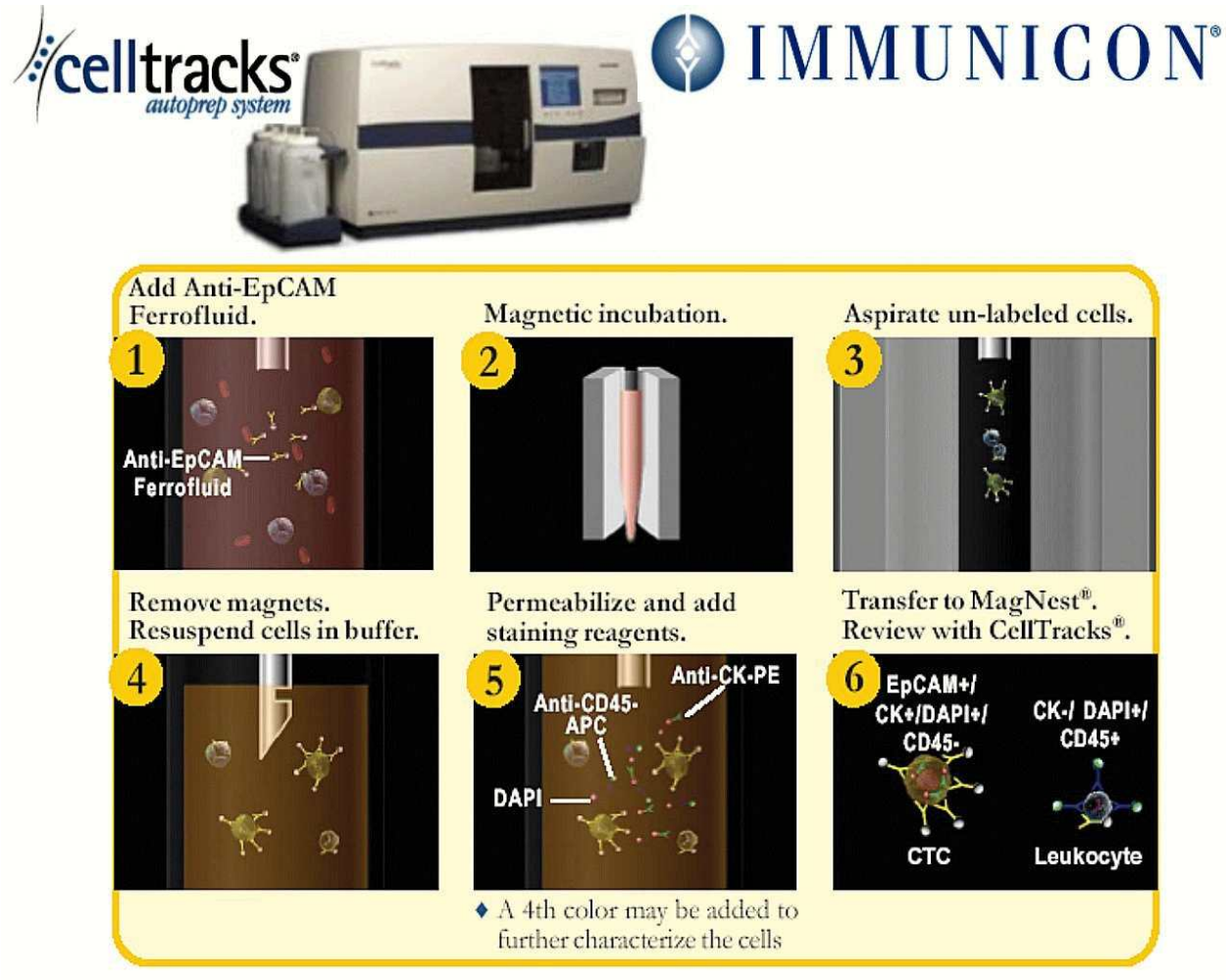
HSV-2 grown on A549 cell monolayer stained with HSV-2 antibodies labeled with RPE and viewed using a fluorescein filter set.

Slides 32 and 33

Immunicon Corporation kindly granted permission to use the images shown in the next pair of slides. They have developed an IVD assay for the capture, analysis and quantitation of rare, circulating, breast cancer tumor cells (CTC's) from blood.

Immunicon's CellTracks® AutoPrep System is an automated sample preparation system for the immunomagnetic capture and fluorescence staining of CTC's based on their expression of a set of specific markers. Immunicon's CellTracks Analyzer II is a semi-automated fluorescence microscope for the enumeration and characterization of the isolated cells.

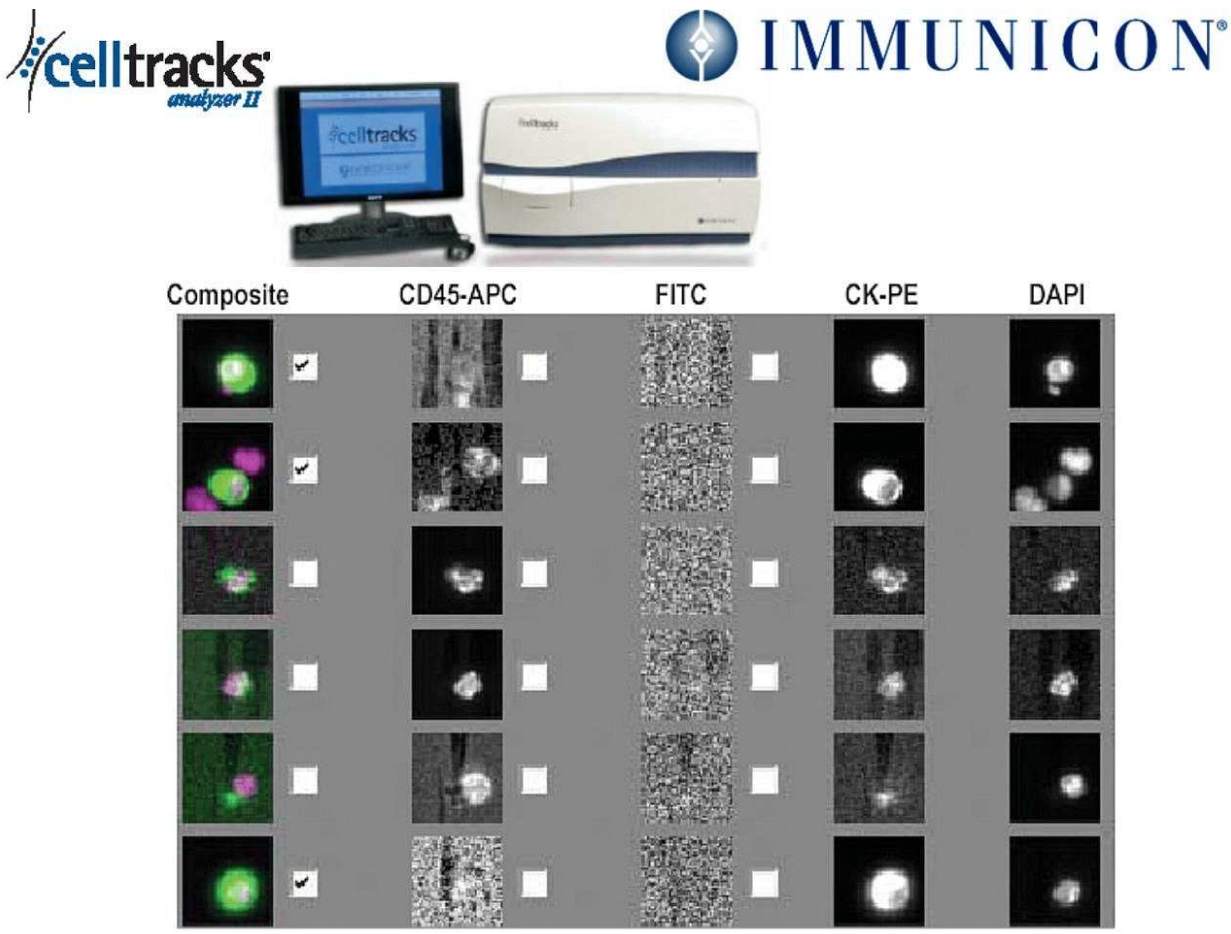
Accurate quantitation and analysis relies on the use of target-specific antibodies conjugated to fluorochromes, including the phycobiliproteins RPE and APC. This is another example of the suitability of this class of pigment molecules for this type of fluorescence application.



Slides 32 - CellTracks® AutoPrep System for Rare Cell Analysis

This slide illustrates the processing steps involved in the capture and staining of CTC's performed by Immunicon's CellTracks AutoPrep System.

The final enriched sample is dispensed into the MagNest® cell presentation device, which presents the captured cells in a single focal plane for quantitative analysis using the CellTracks Analyzer II.



Slide 33 - CellTracks Analyzer II for for Rare Cell Analysis

Sample image gallery obtained with the CellTracks Analyzer II.

Discrimination of CTC's from leukocytes is made possible by the use of lineage-specific markers: cytokeratin-RPE (CK-PE) identifies CTC's, while CD45-APC, identifies leukocytes.

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Slide 34 - Trademarks and Licenses

We show trademarks and licenses which may have been mentioned during the course of the presentation.

Thank you to our PhycoLink customers who provided data for inclusion in this presentation:

Phillippe Poussier, M.D., Associate Professor, Departments of Medicine and Immunology
Sunnybrook Health Sciences Centre

Jimmy Page, Ph.D., Manager, Immunological Detection
Diagnostic Hybrids, Inc
E-mail: jimmy_page@dhiusa.com

Immunicon Corporation
E-mail: sales@immunicon.com

Slide 35 - Conclusion

We've come to the end of our time, so I'd like to thank you once again for coming and I'll now open the floor up to any questions.

ProZyme customers are an important source of information regarding advanced or specialized uses of our products. We encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

TOLL FREE **(800) 457-9444** (US & CANADA)
PHONE **(510) 638-6900**
FAX **(510) 638-6919**
E-MAIL **info@prozyme.com**
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