# Novel strategies for assaying recombinant antibody function with high-throughput cell-based assays

**Christopher Hanna, Doug Gjerde, Liem Nguyen, David Burge and Jeremy Lambert, PhyNexus, Inc. San Jose, CA**
**Tara Arvedson, Amgen, Inc. Thousand Oaks, CA**

## Introduction and Background

Phage and related display technologies enable screening of vast repertoires of recombinant human antibodies for isolation of cognate binders to putative disease targets. Screening operations are typically performed on the basis of antibody-target affinity, with subsequent winnowing of candidates on the basis of other antibody-target binding characteristics (such as its ability to prevent native ligand binding). Once the number of antibody candidates is substantially reduced, more descriptive biofunctional data – which ultimately provides the biological basis of antibody potency – are obtained from more descriptive cell-based assays.

As it has been demonstrated that antibody features such as affinity provide a highly unreliable proxy for antibody potency[1-3], there is growing need to gain more descriptive potency-correlated (i.e. cell-based) data on individual antibody clones earlier in the antibody discovery process. However, there are a number of factors that confound attempts to obtain this data, particularly in the earliest stages of *E.coli*-based antibody discovery. For example, the direct analysis of *E.coli*-derived recombinant antibody sub-clones is confounded by endotoxins and other contaminants intrinsic to the gram-negative bacteria used in the antibody expression process. In addition, the levels of antibody present in *E.coli* preparations are typically of insufficient concentration for use in most cell-based assays. These limitations are most frequently addressed by scaling up the individual growths and using larger-format protein separation and purification tools that do not lend themselves to high-throughput automation – thus creating the present situation where cell-based assays are performed later in the discovery process.

In this presentation we describe how PhyNexus, Inc. PhyTip™ column technology is applied as a high-throughput means for purification and enrichment of recombinant antibodies from small-scale *E.coli* sub-clone preparations so as to eliminate the need for scale-up, and thus facilitates true high-throughput cell-based assays of recombinant antibody function. This capability is demonstrated for cell-based assays of antibody antagonist activity via Stat-dependent luciferase reporting of Stat5 phosphorylation. Factors that contribute to PhyTip column and cell-based assay performance are discussed.

## Phage display procedures

scFv antibody libraries derived from human B cells were prepared and screened by phage display as described elsewhere[4]. Phage libraries were screened with standard panning procedures against the receptor extracellular domain of interest. Upon isolation of the individual antibodies that bound the target, each was subjected to a 2 mL overnight growth at 37 C.

Periplasmic preps were then prepared by centrifuging the culture and resuspending in 300 μL cold TES buffer (50mM Tris-Cl pH 8, 1mM EDTA, 20% sucrose) with protease inhibitors added, and then incubating on ice for 30 minutes. Cell debris was spun down and then combined with 300 μL 1x PhyNexus Capture Buffer (10mM $NaH_2PO_4$ pH7.4, 5mM imidazole, 300mM NaCl).

## Antibody enrichment and purification

A PhyNexus ME or MEA Personal Purification System was used for control of sample processing with PhyNexus PhyTip™ columns (Figures 1 & 2). 200+ Ni-NTA columns (5 μL resin volume) were programmed to perform 10 capture cycles at 0.25 mL/min. This was followed by 2X 200 μL wash cycles of PhyNexus IMAC Wash Buffer 1 (10mM $NaH_2PO_4$ pH7.4, 20 mM imidazole, 300mM NaCl), and 2X 200 μL wash cycles of PBS. Elution was achieved with 20 μL of a low-pH 100 mM phosphate buffer and was neutralized with 100 mM phosphate buffer at pH 9.2.

**Figure 1. PhyNexus PhyTip columns, 200+ and 1000+ sizes**

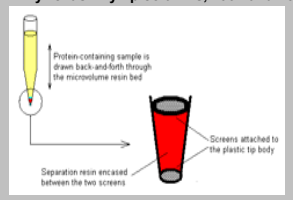


**Figure 2. Design of PhyNexus PhyTip columns**

## Enrichment of antibodies with low-pH elution

Cell-based assays by their nature are sensitive to the chemical environment to which they are subjected. Many Ni-NTA purification and enrichment procedures typically recommend high concentrations of imidazole (200-500 mM) for elution of the his-tagged construct, as this allows for elution under neutral conditions. However, imidazole has a highly toxic effect on many cell types which precludes its use within cell-based assays.

Since the antibodies investigated can tolerate low-pH conditions, acid-based elution was investigated for the purposes of placing the processed antibody in an environment that is more conducive to maintenance of healthy cell conditions. In particular, a phosphate-based buffer system was investigated due to phosphate being generally acceptable to cellular environments. A his-tagged ubiquitin standard was used for these investigations, and it was shown at pH 3 that a concentration of 100 mM phosphate provides high yields that are only marginally improved at higher concentrations (Figure 3). In addition, it was shown that NaCl was necessary for efficient elution of the antibody from the Ni-NTA resin. 100 mM NaCl provided high yields that are only marginally improved for higher concentrations (Figure 4). So as to avoid excessive osmolarities, it is recommended that phosphate concentrations of 100 mM and NaCl concentrations of 50 mM are applied.

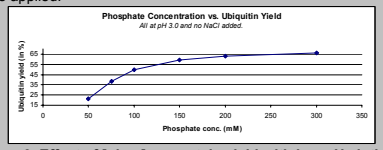


**Figure 3. Effect of [phos] on protein yield with low-pH elution**

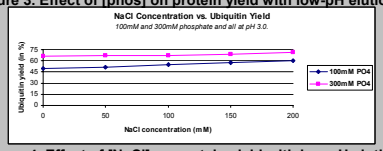


**Figure 4. Effect of [NaCl] on protein yield with low-pH elution**

## Cell-based assays and results

Cell-based assays were established in 32D cells. Inhibition by the antibody of receptor response was determined via Stat-dependent luciferase reporting of Stat5 phosphorylation (shown below in Figure 5). Baseline luciferase reporting signal was established with cells in the presence of a fixed concentration of appropriate growth factor, while the baseline signal absence was established with the same cells in the absence of any growth factor at all.

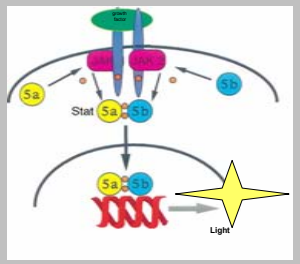


**Figure 5. Cell-based assay for Stat5 phosphorylation via luciferase**

Neutralized antibodies processed as described earlier were added to the cell-based assay and brought to a final volume of 50 μL. Different volumes of prepared antibody were added to the assay and it was shown that up to 4 μL could be added without any deleterious effects on overall healthiness of the cells. In addition, the results obtained for these volumes were highly consistent when comparing different clones (Figure 6). When comparing the baseline luciferase reporting signal (yellow bar, "GF" for *growth factor*) to baseline signal absence (pink bar, "No GF" for *no growth factor*) it is apparent that the different antibody clones examined (clone numbers shown in the legend at right) have demonstrable inhibitory effect, and are known to have inhibitory activity as whole IgGs. In addition, a known non-binder ("NC" for negative control antibody) has the same effect as having GF present without inhibitor. Furthermore, one clone that was known to be an inhibitor (#71, purple bar) gave rise to a signal that was not expected. Upon further examination it was determined that no protein was detected within the periplasmic prep, indicating that this particular antibody did not express properly.

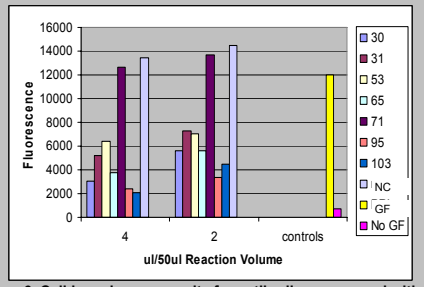


**Figure 6. Cell-based assay results for antibodies processed with Ni-NTA PhyTip columns; low-pH elution with neutralization**

## Conclusions

Cell-based assays represent a powerful generic approach to gaining high-value biofunctional data in a manner that is amenable to highly parallel data collection. Up until now it has been extremely difficult to exploit this technology in a highly parallel manner within the realm of antibody therapeutics, in large part due to the inability to practically and cost-effectively purify and enrich the small quantities of antibody required for these assay formats. PhyTip column technology has shown to be a facilitating tool for the requisite up-front preparation of recombinant antibodies prior to high-throughput cell-based assays, thus providing increasingly descriptive biological information earlier in the antibody discovery process.

## References

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