

High throughput 96-well purification of biopharmaceuticals for cell-based screening

Mike Jones, Debbie Pattison, Mark Liddament, John Andrews, Ruth Franks, Caroline Russell, Stephen Clulow
Cambridge Antibody Technology, Milstein Building, Cambridge, CB1 6GH, UK

Introduction

Cambridge Antibody Technology (CAT) uses its proprietary Phage Display and Ribosome Display technologies to isolate therapeutic antibodies rapidly. Its human antibody libraries contain up to 10^{14} entities that are selected against a target antigen, producing approximately 10^5 samples for screening. Screens use biochemical and cell-based assays to identify biologically active antibodies, most with homogeneous or heterogeneous non-radioactive assays (Figure 1).

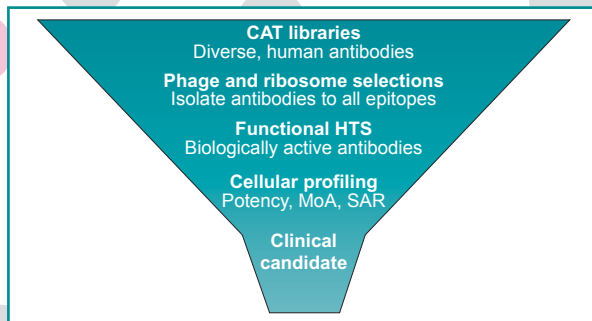


Figure 1. The CAT drug discovery process

Benefits of cell-based screening

The major advantage of an *in vitro* cell-based assay is its potential to distinguish the biologically relevant functional antibodies from the biologically inactive ones. Where a cell-based assay has been used as a primary screen, a higher percentage of hits confirm activity in the secondary screen meaning there is a significant reduction in attrition rates (Figure 2).

Binding	Affinity	Function
Traditional approach	Perceived importance	Functional antibodies
Binding ≠ function	High affinity ≠ high potency	Potential drug leads
Drugs need function	Biological activity more important	More efficient screen
Inefficient 1y screen	Affinity relevant in lead optimisation	Larger lead panels
High "binder to lead" attrition		Lower "hit to lead" attrition

Figure 2. Effective antibody screening

Cell-based assays are sensitive to contaminants, but removing these contaminants is usually a resource-intensive process. In order to allow cell-based primary screening, a high throughput method of purifying samples is essential. These purified samples can also be quantified allowing the clones to be ranked by potency.

The process

The purification method is based on a tip-based purification technology: PhyTip™, developed by PhyNexus (Figure 3).



Figure 3. PhyNexus PhyTip columns, 200+ and 1,000+ sizes

Samples are prepared so that the biomolecule of interest is released into the supernatant. The biomolecule is captured on the resin at the end of the PhyTip as the sample is aspirated. The resin is then washed and the biomolecule eluted.

There are a variety of resins and volumes available: a 20 µl resin provides enough material for testing in a cell-based assay and to determine concentration using a HTRF® assay.

A low pH elution followed by a neutralisation step has been developed, which delivers a maximum amount of biomolecule in a buffer that is well tolerated in a number of cell-based assays. The buffer is effective for eluting scFv from the IMAC matrix and also IgG and Fc fusion proteins from the Protein A matrix. After purification, samples are stored at -70 °C and have been shown to retain activity for at least 2 weeks.

The process has been automated on a Perkin Elmer MiniTrak™, allowing unattended purification of samples.

Using this high throughput small scale purification process, ~2,000 samples can be produced per week. This compares to the large scale purification process, which has a maximum throughput of 192 samples per week.

Reproducibility

Reproducibility of process

The process has been developed to be robust and reproducible. The gel images show the same clones purified on different days. The level of expression is different between runs but the relative expression level of clones is identical (Figure 4).

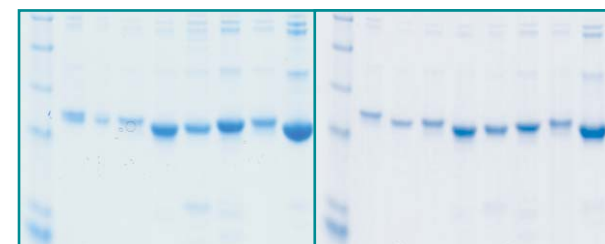


Figure 4. Gel images showing reproducibility of process

Reproducibility between new and existing process

A panel of clones was purified by both the existing large scale scFv purification process and the small scale scFv purification process. The concentration of the scFv was determined and the samples were tested in the same assay. The correlation between the IC₅₀s obtained from the large and small scale scFv purifications was very good (Figure 5). The clone ranking was the same order for both processes. This demonstrates that the small scale purification system can successfully rank and identify clones for further characterisation.

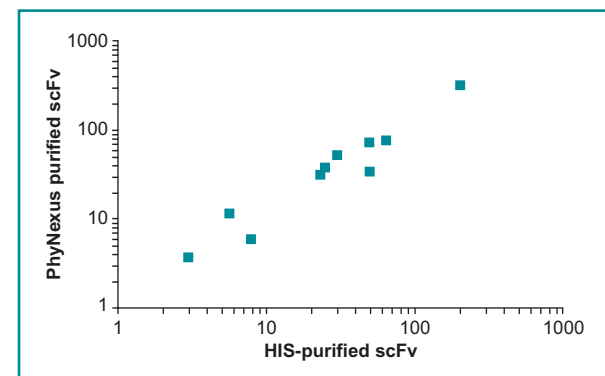


Figure 5. Correlation of IC₅₀s between small scale and large scale purified scFv

Cell assay tolerance

Neutralisation assay

The calcium flux assay shown here utilises a fluorescent dye, which is taken up by the cells. When stimulated by the ligand, the receptor on the cell surface signals and causes a calcium flux. Light is emitted and detected by a fluorescent imaging plate reader. The scFv inhibits the ligand binding to the receptor and the percentage inhibition of signal is measured.

The PhyTips are able to capture and purify enough scFv from a 3 ml culture to give a full inhibition curve. The corresponding periplasmic extract only produces a partial curve (Figure 6).

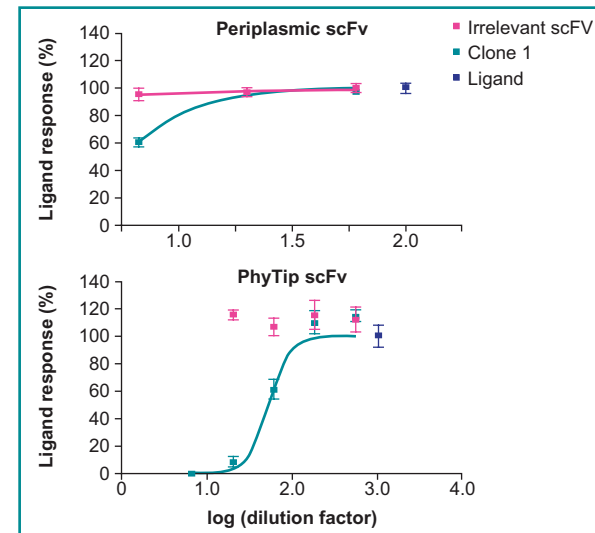


Figure 6. PhyTip purified scFv showing full inhibition curve in calcium flux assay

Reporter assay

In this reporter assay, the ligand binds to the cell surface receptor, activating a signalling pathway. The product of this signalling cascade is secreted into the cell supernatant and measured in a chemiluminescent assay. The scFv binds to the receptor preventing ligand induced signalling.

A significant amount of inhibition was seen on a plate of irrelevant clones when periplasmic extracts were added to the cells. This was due to the by-products produced during the expression of the scFv. By using purified scFv we have significantly reduced the amount of inhibition and variation in the assay similar to that seen on the media control plate (Figure 7).

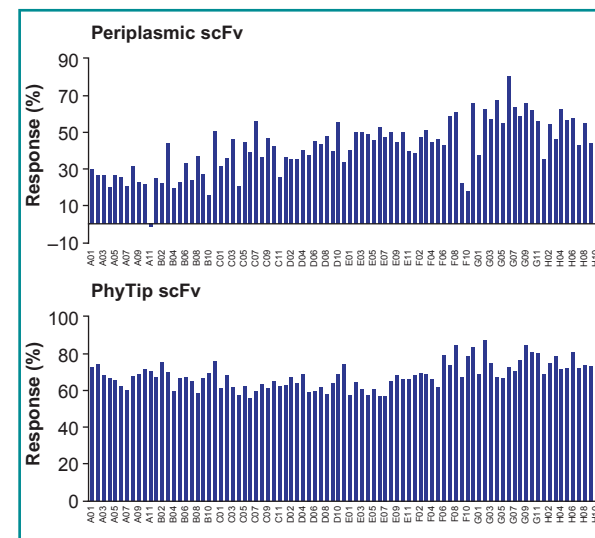


Figure 7. PhyTip purification reduces non-specific inhibition of signalling

Cytokine release assay

This cytokine release assay measures the quantity of cytokine produced by cells in response to a stimulatory molecule using a HTRF cytokine quantification kit. On addition of periplasmic material, a non-specific stimulation of the cells occurred leading to a release of cytokine. This was due to the levels of endotoxin in the sample. By purifying the scFv, the amount of endotoxin in the purified sample was reduced significantly and so did not induce a non-specific response (Figure 8).

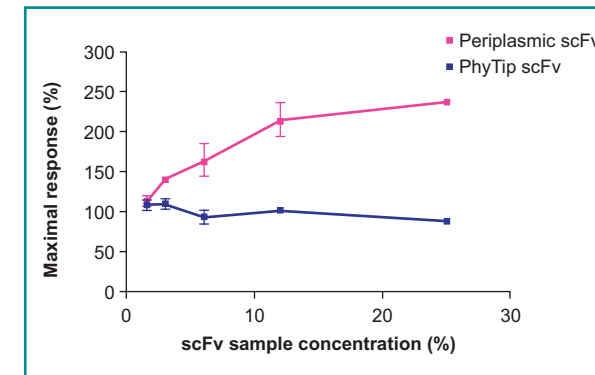


Figure 8. PhyTip purification reduces non-specific stimulation of cytokine release

Dual cell assay

This dual cell assay measures the quantity of cytokine produced by Jurkat cells when stimulated by Raji cells, in the presence of phytohaemagglutinin. Cytokine levels are determined by a europium-based quantikine assay.

Unpurified Fc fusion protein variants expressed in mammalian cells were not tolerated in the assay due to the by-products of expression. These samples were purified from the cell supernatant and their ability to inhibit cytokine production was measured. Purified samples showed expected responses in the assay with no effects from contaminants (Figure 9).

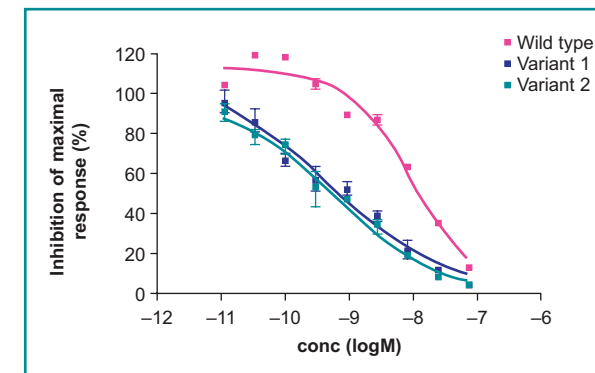


Figure 9. PhyTip purification allows screening of Fc fusion proteins

Quantification for IC₅₀ determination

The samples are measured using a HTRF assay in a 384-well format to reduce the amount of sample needed for quantification. This allows ranking of clones by potency. Only the most potent are purified at the larger scale for further characterisation. The benefits of this are that fewer large-scale HIS preps are required, and more samples can be tested in a cell-based primary screen.

HTRF is a technology developed by Cisbio International and is based on FRET (Fluorescent Resonance Energy Transfer) between a europium (Eu³⁺) cryptate donor and a second fluorescent label acceptor XL665, a stable cross linked allophycocyanin. When in proximity, FRET takes place from the europium cryptate to the XL665, resulting in the emission of an amplified long-lived fluorescence signal.¹

ScFv HTRF method

This assay format is based on a competition between an N-terminally biotinylated tagged c-myc peptide (GAAEQKLISEEDLN) and the scFv (6HIS-c-myc), versus the anti-c-myc monoclonal antibody 9E10 that is conjugated with europium cryptate (Figure 10). The binding signal is inversely proportional to the concentration of scFv.²

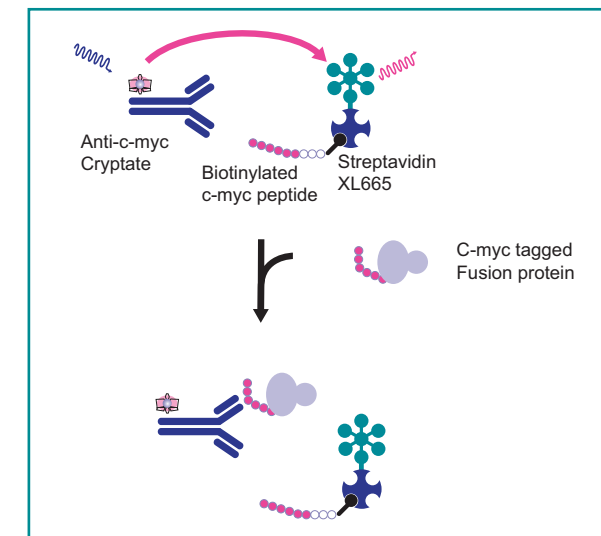


Figure 10. ScFv quantification HTRF assay format

Fc and IgG HTRF method

This assay format is similar to above; the FRET occurs between an XL665-labelled human IgG and a europium cryptate conjugated anti-Fc monoclonal antibody. The IgG or Fc fusion protein to be quantified competes with the XL665 labelled IgG. The binding signal is inversely proportional to the concentration of IgG/Fc fusion protein.³

Summary

At CAT, a high throughput small scale protein purification process has been developed which is robust and reproducible. It allows cell-based primary screens to be run routinely. Previously this had not been possible due to the high level of deleterious by-products from mammalian and bacterial cells. Knowing the concentration of the purified sample allows clones to be ranked by potency. The most potent are purified using the large scale process to provide more material for further characterisation. This decreases the number of resource-intensive large scale HIS preps required for Lead Isolation and Lead Optimisation projects.

Conclusion

This high throughput purification system for biopharmaceuticals allows cell-based screens to be used as the primary assay, which impacts the discovery process as follows:

1. Reduces attrition rates in Lead Isolation
2. Enables the earliest possible identification of agonist scFv, IgG and Fc fusion proteins
3. Allows more rapid identification of the most potent leads in Lead Optimisation
4. Reduces project timelines

References

1. Mathis G. Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. *Clin Chem* 1995;41:1391-1397.
2. Degorce F, Achard S, Coeur B, *et al*. HTRF® check kits for GST, 6-HIS, c-myc and Flag tag accessibility. SBS 5th Annual Conference, September 1999, Edinburgh (UK).
3. Jaga D, Degorce F, Seguin P, Mathis G. New Screening and titration of human Fc chimera using TRACE®/HTRF® technology. SBS 7th Annual Conference, September 2001, Baltimore (USA).

