The complete guide to multiplex assays in drug discovery

Everything you need to know when choosing a suitable multiplex immunoassay for your drug discovery program

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Introduction

What does the guide cover?

This guide aims to highlight the value of multiplexing in drug discovery and assist in choosing the most suitable multiplex immunoassay for your drug discovery program.

The first chapters discuss the advantages of multiplexing and its value for drug discovery. The following chapters highlight the key factors to consider when choosing a multiplex assay and provide a comprehensive overview of various multiplex assay techniques used in screening.

The advantages of multiplex immunoassays

Accurately quantifying protein levels is essential in research and diagnostics, whether quantitatively analyzing the concentration of a particular target or qualitatively testing for the relative presence of protein markers. Despite the robustness and popularity of assays measuring a single analyte (ie, singleplex, such as ELISA or western blot), they may not be the optimal choice for your research as it’s often more biologically informative to analyze more than one target protein simultaneously.

Measuring multiple targets by ELISA involves performing several assay workflows in parallel, which is time-consuming and increases the risk of error. Moreover, sample volume requirements increase with the number of analytes measured, which is a crucial challenge with scarce and precious biological samples.

Multiplex immunoassays enable us to measure multiple target analytes in a single reaction volume reducing workflow and sample volume problems. For example, 25–50 µL sample volume is required to test multiple markers compared with 100 µL per target required for ELISA.

The importance of multiplexing for drug discovery

The demand for safe and efficacious drugs is steadily growing, increasing the need for fast, efficient immunoassays for drug discovery and development. The drug discovery process often starts with lead optimization using high-throughput screening (HTS) to narrow down huge compound libraries to a few promising candidates. HTS requires scalable, high-quality immunoassays to identify the candidates quickly, efficiently, and with minimal costs.

Single analyte immunoassays, such as ELISA, were historically used and are still invaluable in early discovery and measuring therapeutic responses in clinical trials. However, they are inefficient in terms of cost, labor, time, and sample volume required when measuring more than one target protein in HTS. Multiplex immunoassays address those challenges by studying several targets in a single well using minimal amounts of sample and exponentially increasing the number of data points collected in HTS.

Tackling more complex conditions such as cancer and autoimmune disease using increasingly advanced techniques has led to more demand for multiple analyte analyses. For this reason, multiplex assays have become a necessary part of drug discovery as they help identify valid drug candidates earlier in the screening process. Multiplexing achieves this by generating more biologically relevant data from limited sample volume, as various plausible targets are monitored in a single well, eliminating the need for separate sample/control for each target and reducing the coefficient of variability in downstream analysis. Furthermore, multiplex assays can simultaneously look at a target, downstream events, and parallel pathways, thus helping us better identify the participating pathways and players, leading to better drug efficacy. This way, screening can effectively eliminate toxic/ineffective compounds early in the discovery pipeline, decreasing later-stage failures and avoiding costly mistakes1-3.
Choosing a multiplex assay for use in drug discovery

Assay specificity, sensitivity, and reproducibility

Robust analyte specificity is essential when screening thousands of compounds across several targets to find the most promising hits because false positive or negative results may result in wasting time on a false lead or missing a promising drug candidate. Therefore, during screening studies with a high number of samples run in one-shot approaches, specificity outweighs sensitivity, while both high throughput and automation capabilities become extremely relevant.

Assay reproducibility is another critical factor to consider, and it depends on using reliable antibody pairs with high batch-to-batch consistency. Recombinant monoclonal antibodies overcome the issues observed with traditional monoclonal and polyclonal antibodies in terms of consistency between batches and reproducibility. During recombinant antibody manufacture, the antibody genes are cloned in high-yield expression vectors, and the gene sequences used can be modified to improve antibody sensitivity. More importantly, since the antibody gene sequences are controlled, recombinant antibodies are produced with high batch-to-batch consistency, capable of delivering highly reproducible results required by drug development research.1,3

High-quality, validated antibody pairs with:

- Excellent batch-to-batch consistency
- Confirmed target specificity
- High sensitivity
- Automation friendly
- Wash vs no-wash workflow

Low pg/mL Limit of Detection

Assay format:

Figure 1. The main factors to consider when choosing a multiplex assay for use in drug discovery.
Automation

In recent years, automation has revolutionized how the drug discovery pipeline works by streamlining certain decision-making processes\(^4\). As screening libraries contain thousands of candidate molecules, multiple compounds need to be screened in a single assay. To make this possible, automation has been implemented in compound management, HTS and hit-to-lead generation in the last 20-30 years. Since some screening processes are repetitive, monotonous, time-consuming, and prone to error, automation has increased time savings while reducing errors and costs.

In the last ten years, the emergence of robotics, from cherry-picking the compounds to carrying out routine procedures and assays, has transformed the pipeline process. For instance, the use of liquid handlers for dispensing compounds and performing workflows has reduced processing time, decreased the possibility of sample contamination, and increased the efficiency of volume transfers. Advances in automation have encouraged a transition to using smaller volumes of reagents and compounds, with experiments being performed in microtiter plates of high density, usually in a 96-, 384, or 1536-plate format. This process called assay miniaturization has dramatically improved the screening processes by generating large data sets quickly and efficiently while reducing reagent consumption and space\(^5\). Therefore, automation and assay miniaturization have facilitated better-informed decisions and ultimately decreased costs in screening\(^4\).

When performing any HTS study, multiplexing while screening large numbers of samples in a single assay is essential to help identify a few promising lead compounds in a timely and cost-effective manner. Any screening study would benefit from an automation-friendly assay because using liquid handlers/dispensers and robotics will streamline the process, reducing errors in repetitive assays and making screening thousands of compounds smoother and easier.

Wash vs no-wash assay

One of the automation challenges in a multiplex immunoassay is the need for multiple wash steps during assay preparation. Although wash steps reduce the background, increasing the signal-to-noise ratio and thereby sensitivity, they slow down the process and introduce wash step-related errors. In contrast, a no-wash assay decreases the number of steps between reagent handling and final data collection. Transitioning to a non-wash assay workflow simplifies assay setup and automation implementation, which would reduce potential errors and speed up the scale-up during HTS.
Review of multiplex assay techniques employed in screening

Protein identification and quantitation have become extremely valuable in advancing our understanding of disease onset and progression, making the role of robust immunoassays in drug discovery more prominent. A wide range of available assay technologies makes it challenging to determine which platform would be best suited for specific researcher’s needs and study design, allowing to collect the most data in the shortest time. Each assay type has its advantages and disadvantages based on the sample to be profiled and the required sensitivity.

Multiplex immunoassays are divided into two main categories: planar and suspension. Here we describe the key principles, benefits and limitations of planar and suspension assays and review the most common examples of multiplex immunoassays in both categories.

Multiplex suspension assays

In these assays, affinity capture agents are chemically affixed to populations of characterized microscopic coded beads or particles (Figure 2). The subsequent cognate immuno-affinity interactions are detected with fluorescent reporter probes and interrogated by a laser in a flow cell, using flow cytometry principles.

![Planar and suspension assay formats](image)

**Figure 2.** Planar and suspension assay formats. In planar assays, capture ligands are immobilized on a rigid two-dimensional support and probed with a sample. X and Y coordinates are the fixed positions (solid phase) for the binding to occur, with the Z axis resolving the fluorescent or chemiluminescent signal. In suspension assays, capture ligands are immobilized on color- or size-coded microspheres. Suspension assays are distinguished by coding attributes, and flow cytometry is used to detect assay-specific fluorescent signal.
Since the suspension assays use a bead/particle immobilization approach, they need to identify which analyte is being measured on which bead/particle. For this purpose, suspension assays use combinations or levels of dyes to encode analyte-specific capture beads/particles.

**Benefits**
- Flexible assay content

**Limitations**
- Chemical modification to biological capture agents
- Complex instrumentation
- Average sample volume required ~10-12.5 μL

The suspension assay workflow may include multiple wash steps for sensitivity and dynamic range (e.g., Luminex®) or have a no-wash format (e.g., AlphaLISA®). Depending on assay type, multiplex suspension assays can be read on a standard flow cytometer (FirePlex®-96) or require specialized equipment (Luminex®, AlphaLISA®).

**Popular suspension assays:**
- Luminex® technology uses combinations of red and near-infrared dyes to encode solid polystyrene microspheres, potentially generating codes for 500 different targets. These codes are used to identify the bound capture antibodies, and a sandwich assay with phycoerythrin-labeled detector antibodies is used to measure protein levels.

- AlphaLISA® assays use proximity-dependent chemical energy transfer to generate a signal dependent on the occurrence of the desired biomolecular interaction. The multiplexing capabilities with these assays are up to 3-plex and require specific plate readers.

- **Bead arrays** represent a type of multiplex suspension assay that allows simultaneous measurement of multiple analytes using beads containing different concentrations of fluorescent dye. The beads are usually analyzed using standard flow cytometer techniques. The workflow of such assays typically involves multiple wash steps to improve sensitivity and dynamic range.

- **Abcam FirePlex®-96** immunoassays use particle technology for multiplexing protein targets. Affinity agents (antibodies or proteins) are immobilized on proprietary barcoded hydrogel particles, providing the basis for target capture detected through reporter fluorescence to quantify the target. FirePlex-96 measures up to 70 analytes per well, and the readout is achieved on standard flow cytometers.

- **Abcam FirePlex®-384 and 1536** immunoassays use the same particle technology as FirePlex-96® but employ static high content imaging to deconvolute stationary particle codes and analyte-specific detector fluorescence at the bottom of a 384- or 1536-plate well. These assays are compatible with high throughput workflows and instrumentation and can be read with various high content imagers.

**Multiplex planar assays**

In these assays, immuno-affinity capture agents (antibodies or proteins) are affixed co-locally to a planar substrate using various chemical or hydrostatic methods (Figure 2). The subsequent cognate immuno-affinity interactions are detected with fluorescent, chemiluminescent or chromogenic reporter methods and then interrogated with a bi-focal fluorescent laser scanner or CCD camera.

**Benefits**
- Very high-density multiplexing
- Capture agents can be affixed without chemical modification
- Archivable source data
Limitations
- Fixed assay content
- Complex data analysis from large data quantity

Popular planar assays:
- **Plate-based electrochemiluminescence (ECL)** assays use electro-chemiluminescent labels to generate a reporter signal decoupled from the stimulation method, leading to a lower background and higher sensitivity. Measures up to 10-plex per well in a 96-well format but require specific instrument installation.

- **RayBio® antibody array** platform has different antibody array options depending on the studied protein activity and the assay readout.
  - Quantibody® array quantitatively measures proteins using a fluorescent readout.
  - C-Series arrays assess relative protein expression patterns across different sample types and experimental conditions by chemiluminescence.
  - L-Series array allows screening of many proteins per assay for target identification using chemiluminescence or fluorescence.

- **CDI HuProt™** protein microarray profiles proteins immobilized in duplicate on a nitrocellulose-coated or epoxy glass slide. The protein interactions are detected by a biotinylated streptavidin-conjugated fluorescent dye.

**Comparison of standard multiplex assays**

When selecting a multiplex assay, various factors should be considered, such as requirements for specific instrumentation and sample volume, available plate types, plex capability, wash or no-wash workflow, possibilities of automation and high-throughput, and plate re-read option. Table 1 summarizes the main capabilities of the most common multiplex planar and suspension assays.

**Table 1. Technical capabilities of the most common multiplex assays.**

<table>
<thead>
<tr>
<th>Technical requirements</th>
<th>Multiplex Planar</th>
<th>Multiplex Suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Instrument</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Sample volume</td>
<td>10 µL</td>
<td>&lt;10 µL</td>
</tr>
<tr>
<td>Plate type (max wells)</td>
<td>96-well</td>
<td>96-well</td>
</tr>
<tr>
<td>Flex size/well</td>
<td>10</td>
<td>100's</td>
</tr>
<tr>
<td>Workflow</td>
<td>Wash</td>
<td>Wash</td>
</tr>
<tr>
<td>Throughput</td>
<td>Low to medium</td>
<td>High</td>
</tr>
<tr>
<td>Plate re-read possible</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Technical requirements</th>
<th>Bead Arrays Luminex® 100/200</th>
<th>FlexMap 3D® (Luminex®)</th>
<th>FirePlex® 96</th>
<th>FirePlex® 384</th>
<th>FirePlex® 1536</th>
<th>MagPix® (Luminex®)</th>
<th>AlphaLISA®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Instrument</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Sample volume</td>
<td>&lt;10 µL</td>
<td>25 µL</td>
<td>12.5 µL</td>
<td>6.25 µL</td>
<td>2.5 µL</td>
<td>12.5 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>Plate type (max wells)</td>
<td>384-well</td>
<td>96-well</td>
<td>384-well</td>
<td>96-well</td>
<td>384-well</td>
<td>1536-well</td>
<td>96-well</td>
</tr>
<tr>
<td>Flex size/well</td>
<td>12-66</td>
<td>100</td>
<td>500</td>
<td>70</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Workflow</td>
<td>Wash</td>
<td>Wash</td>
<td>Wash</td>
<td>No-Wash</td>
<td>No-Wash</td>
<td>Wash</td>
<td>No-Wash</td>
</tr>
<tr>
<td>Throughput</td>
<td>High</td>
<td>Low</td>
<td>Medium</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Plate re-read possible</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

Learn more at [www.abcam.com/fireplexht](http://www.abcam.com/fireplexht)
Partnering with Abcam on multiplex immunoassay solutions

Multiplex assays allow researchers to simultaneously measure multiple analytes in each well, providing more insightful data to improve decision-making and accelerate research and discovery studies. Abcam offers a comprehensive range of multiplex assays, including high-throughput no-wash automatable formats.

FirePlex multiplex assays

Abcam FirePlex immunoassays have been designed to overcome the common challenges in biomarker and drug screening studies. The FirePlex particle technology (Figure 3) provides multiplex assays with high plex, sensitivity, and high-throughput capabilities.

Figure 3. FirePlex particle technology and its readout on a high content imager. A. Varying intensities of green and yellow fluorescent dyes are used for particle identity barcoding; yellow dye, which is co-locally separate on the particle from the barcoding region, is used for analyte quantitation. B. Two images are captured per well, with approximately 20 particles analyzed per analyte using the FirePlex Analysis Workbench software. C. Representative graph demonstrates analyte fluorescent intensity levels automatically output from scanned wells along with analyte standard curves.

Compared to traditional multiplex assays, FirePlex multiplex assays offer a fast, automatable workflow, small sample requirements, and easy readout on flow cytometers and high-content imagers, saving hands-on time and sample. Furthermore, using our high-quality antibodies with unrivaled reproducibility and excellent sensitivity, FirePlex multiplex assays enable the generation of robust, consistent data.

We offer FirePlex assays for multiplex proteins (FirePlex immunoassays) or miRNA (FirePlex miRNA assays) analysis, including:

- FirePlex-96 immunoassay: up to 70-plex in 96-well plate, ideal for biomarker profiling
- FirePlex-384 and FirePlex-1536 immunoassays: up to 10-plex, or 5-plex respectively, high-throughput (384- and 1536-well) formats with no-wash, automated workflow
- FirePlex miRNA assays: miRNAs profiling directly from small amounts of biofluid or FFPE, without RNA purification or pre-amplification steps

Learn more at www.abcam.com/fireplexht
Fireplex-96 immunoassays

Fireplex-96 immunoassays are fully customizable and highly sensitive multiplex panels enabling analysis of up to 70 analytes per well in a 96-well plate on standard flow cytometers. These immunoassays enable comprehensive biomarker profiling directly from biofluids.

- 0.5 pg/mL to 30,000 pg/mL detection
- Up 5-log dynamic range
- Only 12.5 µL of sample input, direct from biofluids
- Free data analysis software

Learn more about the advantages of Fireplex-96 immunoassays.

High-throughput Fireplex immunoassays

High-throughput Fireplex-384 and Fireplex-1536 immunoassays are ideal for discovery, screening, and profiling projects providing a fast, easy and cost-effective tool to analyze thousands of samples in a fraction of time.

Table 2. Fireplex-384 and Fireplex-1536 capabilities

<table>
<thead>
<tr>
<th></th>
<th>Fireplex-384</th>
<th>Fireplex-1536</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiplex capabilities</td>
<td>10 analytes/well</td>
<td>5 analytes/well</td>
</tr>
<tr>
<td>Assay format</td>
<td>384-well plate</td>
<td>1536-well plate</td>
</tr>
<tr>
<td>Data points per plate</td>
<td>3,840</td>
<td>7,680</td>
</tr>
<tr>
<td>Simple, no-wash workflow</td>
<td>two-step</td>
<td>one-step</td>
</tr>
<tr>
<td>Automated workflow with a readout on high-content imagers</td>
<td>&lt;20 min scan per plate</td>
<td>&lt;1 hour scan per plate</td>
</tr>
</tbody>
</table>

By providing a new, more effective biomarker analysis workflow (Figure 4, 5), Fireplex immunoassays enable data-driven R&D to guide the most effective strategies and minimize the risk of late-stage failures.

Figure 4. The Fireplex-384 simplified, no-wash assay workflow.
Figure 5. An assay workflow of FirePlex-1536, the first 1536-well assay that enables up to 5-plex.

Find out more about high-throughput FirePlex multiplex immunoassays.

How can Abcam help you choose the right multiplex immunoassay for your workflow?

FirePlex immunoassays are available in three different formats to provide the flexibility to build an assay that best meets your needs.

- Choose from ready-to-use, pre-designed panels:
  - Standard FirePlex-96 panels
  - Standard FirePlex-384 panels

- Select analytes of interest from existing panels with Mix and Match assay designer
Case study
Profiling of secreted proteins from iPSCs with FirePlex®-384

From the paper:
Large-Scale Production of Human iPSC-Derived Macrophages for Drug Screening

Simon Gutbier1, Florian Wanke1, Nadine Dahm1, Anna Rümmelin1, Silke Zimmermann1, Klaus Christensen1, Fabian Köchl1, Anna Rautanen1, Klas Hatje1, Barbara Geering1, Jitao David Zhang1, Markus Britschgi1, Sally A Cowley2, Christoph Patsch1,3

Affiliations
1. Roche Pharma Research and Early Development, Roche Innovation Center Basel, Switzerland.
2. James Martin Stem Cell Facility, Sir William Dunn School of Pathology, University of Oxford, UK.
3. BlueRock Therapeutics, New York, USA.

Summary

FirePlex multiplex immunoassays provide a no-wash, automation-friendly workflow in 384- and 1536-well high-throughput formats. Utilizing your existing high-content imager and powered by batch-to-batch consistent recombinant antibodies, FirePlex enables you to achieve high-throughput without sacrificing assay performance or data quality. In this study, a large pharmaceutical company used FirePlex-384 to characterize iPSC-derived macrophages for use in drug screening.

FirePlex®
Multiplex Assays

Power your progress with more insightful data

See how you can accelerate your next discovery
www.abcam.com/fireplexht

For more information: https://pubmed.ncbi.nlm.nih.gov/32645954
Characterization of iPSC-derived macrophage cell line using FirePlex

- Macrophages are used in drug discovery, but their quantities are limited.
- Induced pluripotent stem cells (iPSCs) were utilized to produce a new cell line with a macrophage-like phenotype.
- FirePlex-384 was used to profile the cytokines secreted from the newly developed cell line to confirm its resemblance to macrophages.

Cytokine release of cells derived from suspension storage and directly differentiated after harvesting in unstimulated state and stimulated with 100 ng/mL lipopolysaccharide (LPS) for 18 h was assessed.


- The researchers were able to show that the generated iPSC-derived macrophages closely resemble primary macrophages.
- The iPSC-derived macrophages recapitulate key functional characteristics, including cytokine release, phagocytosis, and chemotaxis.
- The approach used in this study can be helpful to any researchers who need to make detailed characterization and multi-phenotypic profile of any cell type that they isolate, generate, or engineer.

For more information: https://pubmed.ncbi.nlm.nih.gov/32645954
References


