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iQue® Mouse IgG Type and Titer Assay Kit: A Novel Solution to Expedite Antibody Discovery

Zhaoping Liu and John O'Rourke
Essen BioScience, Inc., Part of the Sartorius Group, 5700 Pasadena Ave. NE, Albuquerque, NM 87113 USA

Introduction

With breakthroughs in molecular engineering and antibody humanization, monoclonal antibodies (mAb) are one of the fastest-growing classes of biopharmaceuticals for multiple clinical indications including cancer, cardiovascular disease, autoimmune disorders and infectious disease. Most therapeutic antibody candidates are initially generated using hybridoma technology or primary B cell screening after antigen immunization. In the antibody discovery workflow, primary screens identify clones with specific attributes, i.e., binding specificity, cross species reactivity, selectivity and affinity. Potential clone candidates from the screen are assessed for a variety of critical parameters, such as IgG isotyping, antibody quantification, and cell number | health which is vital information for lead molecule generation (Figure 1).

Quantification of mouse antibody from cell culture supernatant is traditionally assessed using enzyme-linked, immunosorbent assay (ELISA). ELISA is a time consuming, single-endpoint assay, often requiring sample dilution and multiple washes. Additionally, separate IgG isotyping, cell count | health assays are performed to provide the scientific insight needed to facilitate downstream antibody cloning. Sartorius has developed a novel solution to disruptively improve this traditional workflow. The iQue® Mouse IgG Type and Titer Kit is a patented high throughput, multiplexed assay with a wide dynamic range requiring no sample dilution or wash steps. The simple mix-and-read workflow simultaneously measures five endpoints:

- IgG isotype
- IgG quantity per isotype
- Total IgG secretion level
- Cell count
- Cell health

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The high-content data enables quick differentiation between monoclonal and polyclonal wells; determines IgG quantity for qualifying stable clones and for downstream functional assays; provides isotype information to facilitate primer design for PCR-mediated gene cloning; and monitors cell proliferation and health prior to RNA isolation.

The iQue® Mouse IgG Type and Titer assay is part of the iQue® integrated antibody discovery platform technology. The iQue® Advanced Flow Cytometry Platform can acquire data in as little as 20 minutes per 384-well plate, while the iQue Forecyt® software provides powerful data mining tools for large hybridoma | B cell screening studies. The iQue Forecyt® Panorama feature provides dynamic multi-plate data analysis including profile maps with user-specified criteria to quickly identify samples containing the desired IgG isotype, antibody concentration and cell health, critical for analyzing data from large, multi-plate screening campaigns. The adoption of the iQue® Mouse IgG Type and Titer assay can simplify and expedite the antibody discovery workflow, greatly reducing the time to actionable results.

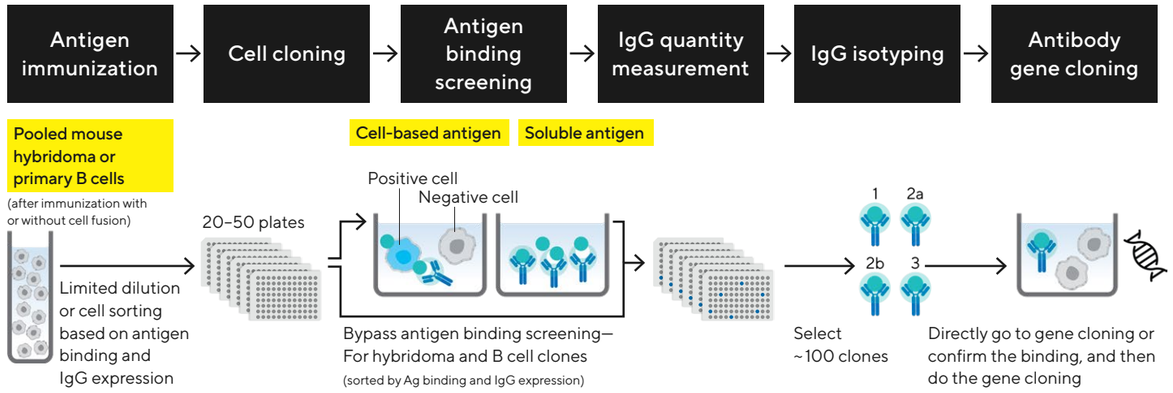


Figure 1: Traditional antibody discovery workflow for lead candidate generation. After antigen immunization, pooled mouse hybridoma cells (post fusion) or primary mouse B cells are grown in microtiter plates using limited dilution or single cell sorting. Primary screening of the cell clones is performed to identify clones with the desired antigen binding characteristics. Positive clones are verified for mouse IgG secretion. Using antigen binding cell sorting methods, single cell clones go directly to the IgG quantification step. Typically, the 100 top positive clones are characterized by IgG isotyping. These samples will be used directly for gene cloning or sequencing. Alternatively, the positive clones are tested in functional assays after normalization of IgG content. After further selection by functionality, the selected cell clones will be used to construct antibody expression vectors.

Materials and Methods

Samples and Reagents

For mouse hybridoma studies, a single plate of 72 hybridoma clones were assessed along with a 12-point IgG standard curve (done in duplicate). Samples containing cell supernatant alone or cell | supernatant mixtures were analyzed using the iQue® Mouse IgG Type and Titer Assay according to the kit protocol.

To assess assay performance in B cell cultures, supernatants from ~ 450 single cell mouse primary B cell cultures were transferred to five, 96-well plates. IgG standard curves were generated on a separate plate. Since B cell samples traditionally secrete much lower levels of antibody than hybridoma cultures, we used the alternative, high sensitivity assay described in the kit protocol.

Data Acquisition and Analysis Workflow

To streamline the analysis process, the assay kit includes a USB drive with a data acquisition and analysis template. This plug-and-play feature includes the plate design, sampling protocol, cell and bead gates, and heat maps. During data acquisition, events populate within the pre-defined gates in the histograms and dot plots in real time. After data acquisition, heat maps and associated metrics are automatically calculated for each corresponding data point. Standard curves to calculate isotype specific and total IgG quantity can be included in the assay plate or in separate plates for analysis. A single standard curve can be applied to all plates in a screening campaign using the multi-plate visualization tool in iQue Forecyt® Panorama.

Monoclonal hits identified by a single IgG isotype and the quantity of each IgG isotype in each sample were identified using the assay template. For multi-plate analysis, profile maps were created with multi-parameter, user defined criteria with the Panorama feature in the iQue Forecyt® software. Multi-plate line graphs and criteria rankings were done using Panorama.

Results and Discussion

iQue® Mouse IgG Type and Titer Workflow

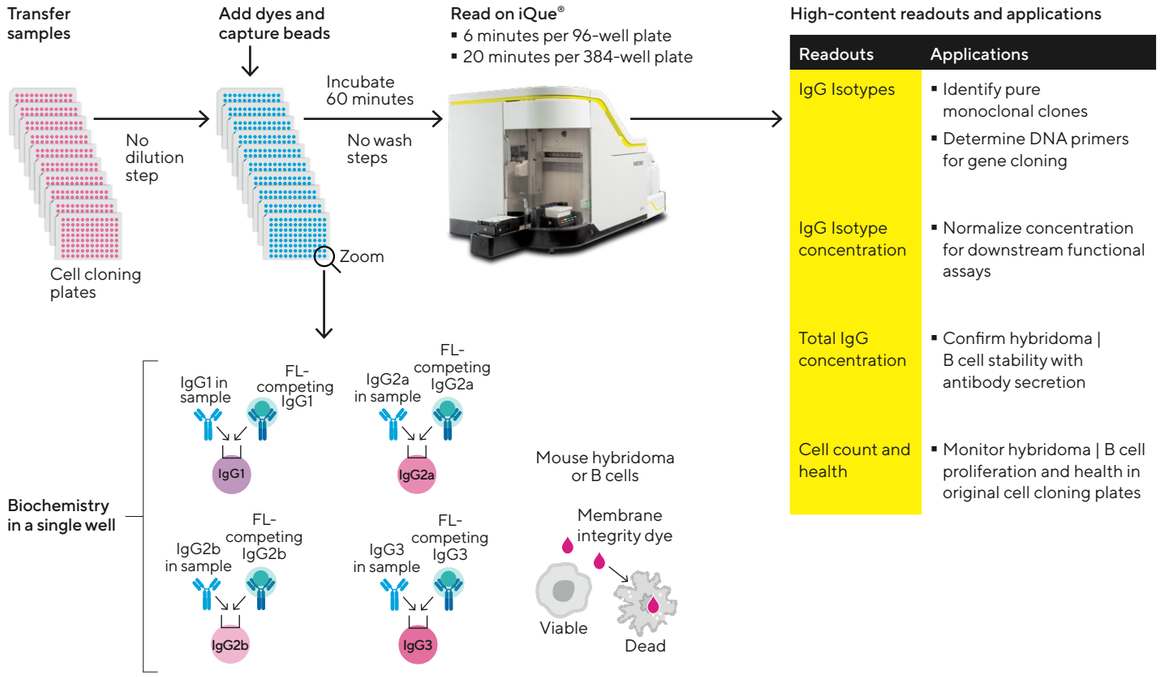
The multiplex assay has a streamlined workflow, which facilitates experimental set-up and data acquisition (Figure 2). Traditional sandwich ELISA has a narrower dynamic range which often requires multiple dilutions of hybridoma culture supernatants to ensure samples are in the linear range of the assay. In contrast, the iQue® Mouse IgG Type and Titer assay has a wide dynamic range (0.05 µg/mL to 50 µg/mL), which encompasses typical hybridoma and B cell culture antibody concentrations. Samples, including cell | supernatant mixtures are directly transferred into assay plates without centrifugation to remove cells and no intermediate labor-intensive dilution steps are necessary. All assay reagents are added to the samples, and no wash steps are required prior to sample acquisition. This mix-and-read format reduces assay set-up time and minimizes the variability associated with multiple washing steps. The total assay set-up time including sample transfer and reagent preparation for a 96-well plate is approximately 15 minutes (only slightly more with a 384-well plate) with an incubation time of one hour. The sample acquisition time for a 96-well plate is 6 minutes (20 minutes using the 384-well plate format). Combined with the plug-and-play data acquisition template, the total time from samples to actionable data is less than 100 minutes.

iQue® Mouse IgG Type and Titer Biochemistry

The iQue® Mouse IgG Type and Titer Kit is a multiplexed competition format (Figure 2). The assay has four different capture beads, with each type of capture bead coated with antibodies specific to a single mouse IgG isotype. The detection reagent is a mixture of four different FITC-labeled mouse IgG proteins. In the sample well, the different IgG isotypes found in the supernatant compete with the corresponding FITC-labeled IgG isotype to bind to the isotype-specific capture bead. Since this is a competition assay, the IgG isotype concentration is inversely proportional to the fluorescence of the isotype-specific capture beads. The sample IgG isotype concentration is calculated from the isotype-specific standard curve. In samples containing both culture supernatant and cells, the cell count and cell viability are also measured.

The unique biochemistry of the iQue® Mouse IgG Type and Titer assays leads to many advantages over traditional methods:

- IgG isotype identification:
  The assay identifies the number of different IgG isotypes from a single clone and thereby detecting polyclonal cultures. This helps guide the decision-making process by identifying monoclonal wells, which may vastly reduce the number of cell subcloning steps, accelerating the antibody discovery process. In addition, isotype information allows for the use of isotype specific DNA primer sets rather than degenerate primers for cloning or sequencing of antibody variable regions.
- Precise total and isotype specific IgG quantification:
  IgG quantity enables normalizing the IgG concentration for all clones, which is necessary prior to downstream confirmatory or functional assays. In addition, the multiplexed isotype-capture bead assay allows more precise quantitation of IgG for each of four isotypes. In singleplex assays such as ELISA, the different IgG isotypes may have different binding affinities to a single "total" IgG capture antibody. Therefore, the generic standard curve cannot precisely measure each IgG isotype in the sample. In contrast, the iQue® Mouse IgG Type and Titer assay uses four different capture beads (IgG1, IgG2a, IgG2b, IgG3), generating four isotype specific standard curves. This leads to precise quantification of each IgG isotype in the sample.
- Simultaneous measurement of cell count and cell viability:
  Monitoring cell proliferation and cell health in the original hybridoma or B cell culture plate is important in determining if the cells are healthy enough for the RNA extraction needed for PCR-mediated antibody cloning.



High-content readouts and applications

Readouts	Applications
IgG Isotypes	▪ Identify pure monoclonal clones ▪ Determine DNA primers for gene cloning
IgG Isotype concentration	▪ Normalize concentration for downstream functional assays
Total IgG concentration	▪ Confirm hybridoma | B cell stability with antibody secretion
Cell count and health	▪ Monitor hybridoma | B cell proliferation and health in original cell cloning plates

Figure 2: iQue® Mouse IgG Type and Titer workflow and assay biochemistry. Samples from hybridoma or B cell cloning plates are directly transferred to the assay plate without an intermediate dilution step. Each assay well is seeded with four different types of capture beads, each with a specific affinity for a single IgG isotype. The IgG isotype in the sample will compete with the same FITC-labeled IgG isotype to bind to the capture bead. The IgG isotype quantity will be calculated from the isotype-specific standard curve. If cells are included in the assay well, a membrane integrity dye in the detection reagent will stain the membrane-compromised dead cells by DNA intercalation. After the addition of the detection reagent mixture and the capture beads to the samples and 60-minute incubation, the samples are acquired on the iQue® Advanced Flow Cytometry Platform.

Data Analysis of a 96-well Hybridoma Plate Using the iQue® Mouse IgG Type and Titer Kit

Traditionally, high-content data requires complicated and time-consuming data analysis. The iQue® Mouse IgG Type and Titer Kit leverages unique iQue Forecyt® analysis algorithms to quickly analyze data and to identify hits. In this study, a single 96-well plate of mouse hybridoma cells were prepared by limiting dilution. After ~ two weeks, 20 µL of sample from 72 clones (containing a mixture of cells and supernatant) were transferred to an assay plate. After incubation with assay reagents, samples were acquired on the iQue® Advanced Flow Cytometry Platform (Figure 3).

The kit template automatically generated four isotype-specific standard curves, and all the sample IgG isotypes were automatically quantified and populated in heat maps and a data grid. Cell count and cell health information was also automatically populated in the same visualization format. Next, an iQue Forecyt® Panorama view of all IgG isotypes in the sample wells were created using an overlay line graph. All 72 mouse hybridoma samples with IgG isotype and quantity information are displayed in the line graph for monitoring and quality-control of the screening results at the single plate level. The total time from data acquisition to the identification of potential candidates for downstream processes only takes minutes per plate.

Data Analysis of a Multi-Plate B Cell Screening Campaign

In this experiment 20 µL of supernatant from five, 96-well plates of primary B cell clones were analyzed using the high sensitivity protocol found in the iQue® Mouse IgG Type and Titer Kit (due to the comparatively low levels of antibody secretion in B cell cultures; Figure 4). Standard curves were generated from a separate standard plate and iQue Forecyt® used these isotype-specific standard curves to auto-calculate the IgG quantity in each sample. The data from the five plates were populated in the data grid in the iQue Forecyt® Panorama feature. Profile maps identifying specific hits at the screening level (all five plates) were produced with user defined criteria (wells that had greater than 0.1 ug/mL IgG1 and less than 0.1 ug/mL of all other isotypes). Samples that met the profile map criteria were ranked by IgG1 concentration using a Panorama line graph. The data acquisition analysis and this whole screening campaign was complete in about one hour.

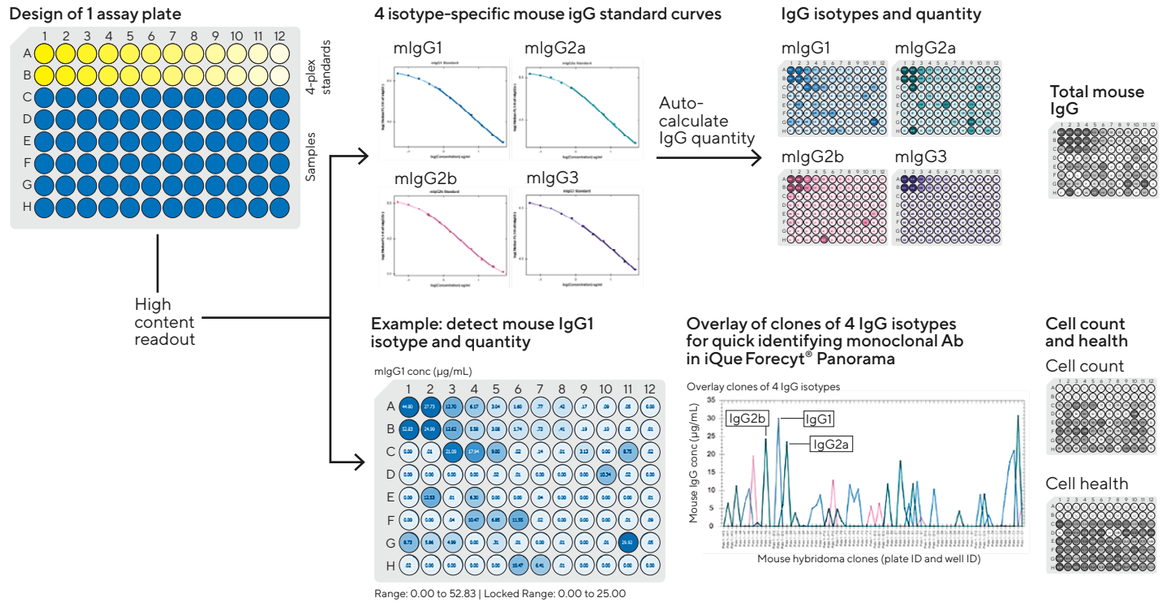
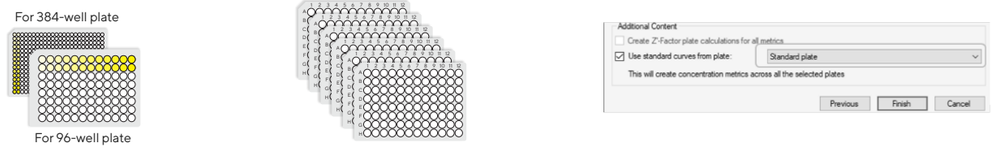


Figure 3: Analysis of high content readouts from hybridoma samples using iQue® Mouse IgG Type and Titer Kit. A single microtiter plate was designed with in-plate 4-plex standards for the top two rows and with hybridoma cell | supernatant samples in the bottom six rows. After data acquisition into the assay template, the analysis algorithm automatically generates four isotype-specific standard curves and IgG concentration is quickly calculated for each sample. The analysis software also provides the IgG isotyping and clone purity information in an overlay line graph in the Panorama function of iQue Forecyt®. Cell count and health | viability is displayed in heat maps or in a data grid.

1. Optional: Run a standard plate on iQue® (Only necessary if no standard in screening plate)
2. Run a full set of screening plates (384-well or 96-well) on iQue®
3. Calculate IgG Concentrate
   - In Panorama in iQue Forecyt® 6.0 and up version
   - Or directly in iQue Forecyt® (4.1 and up if with in-plate standard; 6.2 and up if without in-plate standard)



4. Auto-populate all clones in table with iQue Forecyt® Panorama Data Grid
5. Identify wells with monoclonal with iQue Forecyt® Panorama Profile Map Set thresholds: (B cell cloning example)
   - IgG1 > 0.1 µg/mL
   - Other isotype: < 0.1 µg/mL or other user's defined number
6. Pool monoclonal wells with all plate ID and well ID info in iQue Forecyt® Panorama Line Graph (Use IgG1 monoclonal as example)

Figure 4: Data Acquisition and analysis workflow in a B cell screening campaign using the iQue® Mouse IgG Type and Titer Kit. Supernatants from ~ 450 clones were analyzed in a multi-plate screening study. IgG quantitation at the screening campaign level, and profile maps were generated to identify monoclonal samples containing high levels of IgG1. Samples meeting the user defined criteria were ranked by IgG1 concentration using a line graph.

Conclusion

The iQue® Mouse IgG Type and Titer Kit is a novel assay platform for mouse antibody discovery that disruptively improves the traditional time-consuming workflow by combining three separate assays into a single multiplex, high throughput assay. The competition format enables a wide dynamic range for IgG detection which means no sample dilution and no wash in the assay workflow, reducing variability. The multiplex assay provides high content data to analyze specific antibody cell clone parameters and makes it possible to differentiate samples with high monoclonal IgG secretion with healthy cell proliferation, from polyclonal samples, samples with poor antibody production, and samples containing unhealthy cells. Coupled with the rich visual context provided by iQue Forecyt® software and the multi-plate Panorama analysis algorithm, researchers benefit from a big-picture perspective to quickly identify lead candidates. Leveraging the novel iQue® Mouse IgG Type and Titer assay platform and unique data analysis software, the iQue® antibody discovery solution provides a streamlined workflow to dramatically expedite the time from data to actionable decisions.

North America
Essen BioScience Inc.
300 West Morgan Road
Ann Arbor, Michigan, 48108
USA
Phone: +1 734 769 1600
Email: AskAScientist@sartorius.com

Europe
Essen BioScience Ltd.
Units 2 & 3 The Quadrant
Newark Close
Royston Hertfordshire
SG8 5HL
United Kingdom
Phone: +44 1763 227400
Email: euorders.UK03@sartorius.com

Asia Pacific
Sartorius Japan K.K.
4th Floor Daiwa Shinagawa North Bldg.
1-8-11 Kita-Shinagawa
Shinagawa-ku, Tokyo
140-0001
Japan
Phone: +81 3 6478 5202
Email: orders.US07@sartorius.com

For further information, visit www.sartorius.com

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