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Johns Hopkins University
For research on synaptic vesicle endocytosis

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Analysis of purified PKB activity

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Individual cell analysis for protein expression and cell type quantitative alternative to standard bulk detection methods

Detection and quantitation of proteins in biological samples is an essential task routinely performed in countless disciplines and in laboratories all over the world, for activities ranging from basic protein characterization to clinical diagnostic testing and drug development. Common methods for protein detection include enzyme-linked immunosorbent assay (ELISA), dot blot, and Western blot (also called protein immunoblot).

While these standard assays continue to present a reliable means of cell analysis for thousands of targets, recent advances in instrumentation offer significant improvements in time savings and convenience for common markers. An additional limitation of bulk assays is the need to homogenize cells or tissue, resulting in a loss of information from individual cells in a population. Techniques that detect and report signal from individual cells can provide quantitative data from large populations about protein target or other marker levels, from cells of varying phenotype, developmental state, or health status.

Quantitative data from large populations with single-cell precision

Flow cytometry addresses this need for quantitative data from significant cell populations by interrogating individual cells for the presence and relative strength of signal from fluorescent reagents or antibodies. However, traditional flow cytometers require extensive operator training and expertise, and sheath fluid-based systems are characterized by extensive setup and shutdown—as well as considerable cost to purchase, operate and maintain.

The Muse® Cell Analyzer was developed to give researchers simple, affordable access to the quantitative data that flow cytometry provides for measuring markers of viability, mitochondrial health, protease activity, and more. Built on flow cytometry principles, Muse® uses microcapillary fluidics and pre-optimized reagents to create an inexpensive, compact, portable system that requires little setup and no expertise to operate. These attributes present a rapid, simplified alternative to more time-consuming methods like Western blot (that may also demand considerable technical expertise) for routine analysis of cell culture health, and to assess the effects of compounds for toxicology and drug discovery screening.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Key materials and equipment needed</th>
<th>Hands-on time</th>
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<tr>
<td>ELISA</td>
<td>Coated multiwell plates, Capture antibody, Detection antibody conjugate, Enzyme substrate, Stop solution, Plate washer, ELISA reader</td>
<td>1.5 hours</td>
<td>8 hours – 2.5 days</td>
</tr>
<tr>
<td>Dot blot</td>
<td>Blot cassette, Blocking buffer, Nitrocellulose membrane, Protein standard, Primary antibody, Secondary antibody, Detection reagent</td>
<td>1.25 hours</td>
<td>5.5 hours</td>
</tr>
<tr>
<td>Lysis buffer protein quantification kit, Protein standards, SDS page gels, Electrophoresis chamber, Loading buffer, Running buffer, Transfer buffer, Protein transfer chamber, Membranes, Filter paper, Blocking buffer, Primary antibody, Secondary antibody, Gel/blot imager</td>
<td>2 – 3 hrs</td>
<td>10 hrs – 2.5 days*</td>
<td></td>
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<tr>
<td>Muse®</td>
<td>Muse® Cell Analyzer</td>
<td>10-15 mins</td>
<td>10 mins – 4 hrs.</td>
</tr>
</tbody>
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Table 1. Workflow comparison among protein detection methods. For proteins not already in solution, bulk methods require lysis of samples to render proteins accessible to detection. Reagents, supplies, and equipment for preparation, transfer, probing, detection, and imaging may be needed, depending on technique. Lengthy primary antibody incubations and serial washes following each binding step can result in significant hands-on and total elapsed time, for routine protocols. The Muse® Cell Analyzer uses preoptimized reagent cocktails to minimize variation and instrument setup, resulting in appreciable reductions in time spent on the bench and total start-to-result time. *Significant time savings can be achieved by use of a vacuum-driven method such as the SNAP i.d.® 2.0 system for Western blotting.

Summary of protocol

Culture cells, including negative and positive controls, for time needed to induce apoptosis.

Dilute Muse® 10X Caspase Buffer to 1X with DI water.

Prepare cell samples in 1X Caspase Buffer for incubation with Muse® MultiCaspase Reagent working solution.

Reconstitute Muse® MultiCaspase Reagent with 50 µL of DMSO to make stock solution.

Mix thoroughly and run on Muse® Cell Analyzer

Figure 1. Typical Muse® assay experimental protocol summary. Steps shown are from the protocol for the Muse® MultiCaspase Assay kit (Cat. No. MCH100109) for detection of the activity of caspases 1, 3, 4, 5, 6, 7, 8, and 9. Results from this assay are available in approximately one hour, about 20 minutes of which requires hands-on activity.
Individual cell analysis for protein expression and cell health: a quantitative alternative to standard bulk detection methods

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<tr>
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<td>Protein standards&lt;br&gt;SDS page gels&lt;br&gt;Electrophoresis chamber&lt;br&gt;Loading buffer&lt;br&gt;Running buffer&lt;br&gt;Transfer buffer&lt;br&gt;Protein transfer chamber&lt;br&gt;Membranes&lt;br&gt;Filter paper&lt;br&gt;Blocking buffer&lt;br&gt;Primary antibody&lt;br&gt;Secondary antibody&lt;br&gt;Ge/Blot imager</td>
<td>2 – 3 hrs</td>
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- Reconstitute Muse® MultiCaspase Reagent with 50 µL of DMSO to make stock solution.

Dilute Muse® MultiCaspase Reagent stock solution 1:160 with 1X PBS to make working solution.

Prepare Muse® Caspase 7-AAD working solution by adding 2 µL of 7-AAD to 148 µL of 1X Caspase Buffer.

Add 5 µL of Muse® MultiCaspase working solution to 50 µL of cells

Add 150 µL of 7-AAD working solution

Mix thoroughly and run on Muse® Cell Analyzer

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BS-GEN-16-12413 3/2016 © 2016 EMD Millipore Corporation, Billerica, MA USA. All rights reserved.
Population means vs. individual cell quantitation

Data that can be quantified are increasingly important in the life sciences, as quantitative data are objective and therefore considered more reliable, as well as being subject to statistical analysis. Quantitative data are assumed to be more representative of populations than qualitative data, and therefore must be characterized both by significant sample size and by the capacity to measure individual events in a sample.

Figure 2. Common bulk immunodetection assay results contrasted with quantitative data. A. Enzyme-linked immunosorbent assay (ELISA), left panel, uses a colorimetric or a fluorescent detection reagent. Spectrophotometry can be used to transform signal intensity into numerical values, but signal intensity is a mean from all cells or cell products in a sample, as is the case with dot blot (middle panel). The right panel is an example of fluorescent detection of immunoblot (Western blot), showing the ‘ladder’, or molecular standard, in lane 1. B. Left panel, Western blot of recombinant histone H2A.X (lane 1), recombinant histone H2A (lane 2), and acid extracted proteins from HeLa cells (lane 3) were probed with anti-histone H2A.X. The right panel shows representative data from the Muse® H2A.X activation dual detection assay, which uses two directly conjugated antibodies against the unmodified and phosphorlylated histone target to map signal from every cell in the sample onto a scatter plot. Absolute numbers and percent of cells activated in the sample are automatically calculated and displayed on the ‘Statistics’ tab.

Although spectrophotometry and densitometry can be used to transform sample well color or the size of a blot or band into numerical values for comparison of relative signal intensity among samples, these methods rely on homogenization of all of the cells or tissue in a particular sample. Western blot relies on concurrent electrophoresis of a mixture of proteins of known weight to create a standard, or ‘ladder’ of bands on the blot, to which positive bands from sample lanes are compared for confirmation of the protein’s identity (Figure 2A, right panel). Because no identity information can be gained from immunoblot without the standard for comparison, Western blot is considered ‘semi-quantitative’.

Conclusions

Immunoblot and immunosorbent assays continue to be among the most popular methods for protein detection in the life sciences, as they are amenable to measuring virtually any target for which an epitope binder such as an antibody can be developed. These methods are constrained, however, by the inability to capture population variation due to the homogenization of sample. Standard bulk methods may also not be optimal for routine screening because of the time they consume in the lab and the expertise they require in order to optimize reagents and to obtain, interpret, and troubleshoot results.

Simplified flow cytometry-based analysis presents a rapid, uncomplicated, cell-based alternative to methods such as immunoblot, particularly for routine and frequent screening of cell cultures, or for response of cell models to compounds in development for chemotherapeutics, drug discovery, cosmetics and similar applications. In addition to detecting key protein targets, The Muse® system incorporates assays for detection using familiar cell status indicators that do not rely on antibody-protein interactions, such as fluorescent membrane integrity dyes and nucleic acid binders.

The Muse® software automatically returns cell-by-cell results from these reagents, unlike microscopy or other low-throughput, time-consuming or subjective techniques for measuring their signal. Muse® assays are selected to provide an efficient means for the most essential viability, cell health, and signaling screening, and reagents are pre-optimized to minimize variation and the need for complex setup adjustments that characterize traditional open-system cytometers.

Despite its small size and remarkably simplified operation, the Muse® system returns the same powerful single-cell data as larger, more costly and complex systems. The availability of rapid, quantitative cell analysis without the need for extensive investment in supplies or trained personnel has the potential for significant impact on compound screening and cell culture model paradigms in the pharmaceutical and life science research domains.

To learn more about the Muse® Cell Analyzer and see a complete list of Muse® assays, please visit: www.emdmillipore.com/muse
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