APPLICATION NOTE

Attune NxT Flow Cytometer with Autosampler

High-throughput automation with the Attune NxT Autosampler: consistent results across all wells and across plates

Introduction

The emerging field of high-throughput (HT) flow cytometry is extending the capabilities of cell-based screening technologies into the profiling of compound libraries. The screening of molecules for effects in living cell populations has become an integral step in virtually every drug discovery program. High-throughput flow cytometry is a particularly useful approach for screening compounds in cell models where multiple readouts are desired. This can be performed using the Invitrogen™ Attune™ NxT Flow Cytometer with Attune™ NxT Autosampler, where 96- and 384-well plates can be acquired and analyzed. In addition, up to 16 different parameters per cell can be measured on the Attune NxT Flow Cytometer, and thousands to millions of cells can be analyzed in minutes at up to 35,000 cells/sec and 1 mL/min sample input.

The Attune NxT Flow Cytometer uses a unique volumetric sample and sheath fluid delivery system. Samples are introduced to the Attune NxT Flow Cytometer with syringes, producing accurate measurements of the volumes of acquired samples and thus accurate calculation of cell concentrations. Given time savings, high content, and assay robustness, high-throughput flow cytometry provides the capacity needed as part of an effective and practical systems biology approach. These recent developments expand the role of flow cytometry in drug discovery and life science research and are a key factor in understanding cellular and molecular networks.

In this application note, consistency across 96-well plates was examined. In brief, lysed human whole blood was labeled with fluorophore-conjugated monoclonal antibodies for CD45, CD3, CD4, and CD8 targets. The labeled cells were fixed, then added in equal amounts to each well of two 96-well plates, and acquired on a 4-laser Attune NxT Flow Cytometer with Autosampler. The concentration statistic representative of events/µL of any given population and percent positive are examined in depth.

ThermoFisher
SCIENTIFIC

Materials

- Invitrogen™ CD45 Mouse Anti-Human mAb, Alexa Fluor™ 488 Conjugate (Cat. No. MHCD4520)

- Invitrogen™ CD3 Mouse Anti-Human mAb, APC Conjugate (Cat. No. MHCD0305)

- Invitrogen™ CD8 Mouse Anti-Human mAb, Pacific Blue™ Conjugate (Cat. No. MHCD0828)

- Invitrogen™ CD4 Mouse Anti-Human mAb, R-PE Conjugate (Cat. No. MHCD0404)

- Attune NxT Flow Cytometer, 4-laser standard configuration (Cat. No. A24858)

- Attune NxT Autosampler (Cat. No. 4473928)

- Invitrogen™ AbC™ Total Antibody Compensation Bead Kit (Cat. No. A10497)

- Human whole blood with sodium heparin anticoagulant, collected from a normal donor in compliance with institution-approved policy

- Gibco™ ACK Lysing Buffer (Cat. No. A1049201)

- Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. AMQAX1000)

- Countess Cell Counting Chamber Slides (Cat. No. C10228)

- Invitrogen™ Trypan Blue Stain, 0.4% (Cat. No. T10282)

- Corning™ Falcon™ 96-well U-bottom polystyrene microplates (Fisher Scientific Cat. No. 08-772-54)

- Thermo Scientific™ Pierce™ 16% Formaldehyde (w/v), Methanol-Free (Cat. No. 28906)

- Falcon™ 50 mL Conical Centrifuge Tubes (Cat. No. 1443222)

- 12 x 75 mm polystyrene flow cytometry tubes

- Gibco™ PBS, pH 7.4 (Cat. No. 10010023)

- Gibco™ AlbuMAX™ I Lipid-Rich BSA (Cat. No. 11020021)

The following protocol was used for sample preparation, acquisition, and analysis on the Attune NxT Flow Cytometer. Please see the user guides for detailed instructions on setting up an experiment and running samples [1-3]. Table 1 lists the lasers, detector bandpass filters, and fluorophores used.

Table 1. Instrument configuration and antibody conjugates used.

Target	Fluorophore	Detector	Laser wavelength (nm)	Bandpass filter (nm)
CD45	Alexa Fluor 488 dye	BL1	488	530/30
CD3	APC	RL1	637	670/14
CD4	R-PE	YL1	561	585/16
CD8	Pacific Blue dye	VL1	405	440/50

Methods

1. Red blood cell lysis

1.1 Collect 20 mL of whole blood by venipuncture with sodium heparin anticoagulant.

1.2 To each of four 50 mL conical tubes, add 5 mL of whole blood and 45 mL of 1X ACK Lysing Buffer.

1.3 Incubate at room temperature for 10 min.

1.4 Centrifuge samples at 300 x g for 5 min at room temperature.

1.5 Aspirate the supernatant, leaving approximately 100 µL to avoid disturbing the cell pellet.

1.6 Prepare 4% formaldehyde and PBS with 1% BSA for stock reagents.

1.7 Add 5 mL of PBS with 1% BSA to the cells, and gently mix.

1.8 Centrifuge samples for 5 min at 300 x g.

1.9 Aspirate the supernatant, and resuspend the cell pellet in 2 mL of PBS with 1% BSA and gently mix.

1.10 Combine the samples from all four tubes into one 50 mL conical tube.

1.11 Count cells on the Countess II Automated Cell Counter as described in the instrument user guide [4], or on another counting device.

1.12 Dilute cells to a concentration of 1 x 10^7 cells/mL in PBS with 1% BSA.

2. Antibody labeling and fixation (bulk sample)

2.1 Add 2 mL of cell suspension prepared in step 1.12 to a new 50 mL conical tube.

2.2 Add 60 µL of each of the four antibody conjugates, and mix gently.

2.3 Incubate at room temperature or on ice for 20 min, protected from light.

2.4 Add 20 mL of PBS with 1% BSA to sample, and gently mix.

2.5   Centrifuge sample for 5 min at 300 x *g*.

2.6   Remove and discard supernatant.

2.7   Resuspend cell pellet in 20 mL of 4% paraformaldehyde.

2.8   Incubate at room temperature for 20 min, protected from light.

2.9   Centrifuge sample for 5 min at 300 x *g*.

2.10   Remove and discard supernatant.

2.11   Resuspend sample in 20 mL of PBS with 1% BSA, and gently mix.

3. Preparation of fluorescence minus one (FMO) controls

3.1   Add 100 µL of lysed whole blood (from step 1.12 above) to each of five 12 x 75 mm flow cytometry tubes. An optional additional sample of unlabeled cells may be prepared.

3.2   Prepare FMO controls by adding antibody conjugates and cells to tubes as indicated in Table 2.

3.3   Incubate at room temperature or on ice for 20 min, protected from light.

3.4   Add 4 mL of PBS with 1% BSA to each tube, and centrifuge for 5 min at 300 x *g*.

3.5   Remove supernatant, and resuspend samples in 1 mL of PBS with 1% BSA.

4. Preparation of single-color compensation controls

4.1   Gently vortex the vials of AbC capture beads (Component A) and negative beads (Component B) for 10 seconds before use [5].

4.2   Label a sample tube for each of the four antibodies, and add 1 drop of capture beads (Component A) to each tube.

4.3   Add 5 µL of each individual antibody conjugate to capture beads in the designated tubes (one antibody conjugate per tube), and mix well.

4.4   Incubate for 15 min at room temperature, protected from light.

4.5   Add 3 mL of PBS to each tube, and centrifuge for 5 min at 300 x *g*.

4.6   Carefully remove the supernatant, and resuspend the bead pellet in 0.5 mL of PBS.

4.7   Add one drop of negative beads (Component B) to each tube immediately, and mix well.

4.8   Mix each tube well prior to acquisition by flow cytometry. Perform compensation according to the protocol for the Attune NxT Flow Cytometer using the Negative Gate method. Gate on the bead singlet population on the forward scatter (FSC) vs. side scatter (SSC) dot plot. Record each sample.

Table 2. Preparation of FMO controls.

Sample	Unlabeled cells (from step 1.12)	CD45 Alexa Fluor 488	CD3 R-PE	CD4 APC	CD8 Pacific Blue	Labeled cells (from step 2.11)
Unlabeled sample	100 µL	None	None	None	None	None
FMO, no CD45 Alexa Fluor 488	100 µL	None	5 µL	5 µL	5 µL	None
FMO, no CD3 R-PE	100 µL	5 µL	None	5 µL	5 µL	None
FMO, no CD4 APC	100 µL	5 µL	5 µL	None	5 µL	None
FMO, no CD8 Pacific Blue	100 µL	5 µL	5 µL	5 µL	None	None
4-color labeled sample	None	None	None	None	None	100 µL

5. Flow cytometer setup

5.1 Turn on the Attune NxT Flow Cytometer, Attune NxT Autosampler, and computer; run startup and performance test modules.

5.2 In Experiment Explorer, create a plate experiment with 5 tube samples and 2 plate samples, with 96 samples per plate. Select round-bottom 96-well plate type.

5.3 Using the Parameters tab under Instrument Settings, add the target and fluorophore label name, and deselect fluorescent detectors not being used in the experiment (Figure 1).

Figure 1. Parameters under the Collection Panel.

5.4 Create the following workspace in the Attune NxT Software:

- Plot A: SSC vs. BL1 (CD45 Alexa Fluor 488)

- Plot B: Time vs. SSC

- Plot C: RL1 (CD3 APC) vs. YL1 (CD4 R-PE)

- Plot D: RL1 (CD3 APC) vs. VL1 (CD8 Pacific Blue)

- Plot E: YL1 (CD4 R-PE) vs. VL1 (CD8 Pacific Blue)

- Statistics table, with percent total, percent gated, and concentration (events/µL) selected on the statistics ribbon

5.5 Parent gate for plots A and B is All Events.

5.6 Create a gate around the lymphocyte population in the SSC vs. CD45 plot.

5.7 Parent gate for plots C, D, and E is the CD45+ lymphocyte gate created on plot A.

5.8 Add quadrant gates to plots C, D, and E.

6. Acquisition

6.1 Run and record the single-color compensation controls using the automated compensation module. Adjust gates as necessary on the positive and negative populations for each single-color compensation sample. The compensation is set automatically once all the controls have been recorded, and may be viewed as either the compensation matrix or spillover matrix. The spillover matrix shows the amount of spillover from each fluorophore into each of the other fluorescent detectors. Data compensation is calculated using the compensation matrix, which is the inverse of the spillover matrix. In this experiment, minimal spillover is observed (Figure 2).

Figure 2. Spillover matrix for the four fluorophores used in this experiment.

6.2 Proceed with recording the FMO and gating control samples in tubes, using the same instrument settings for the fluorescent detectors that were used for recording compensation controls. The FSC and SSC voltages will require adjustment to get the white blood cell (WBC) populations on scale. The default FSC threshold was used in this example; this low setting allows the display of debris, distinct from cells.

6.3 In the Collection Panel of Attune NxT Software, set the following run protocol for the 96-well plates (Figure 3):

Figure 3. Collection Panel for 96-well plates.
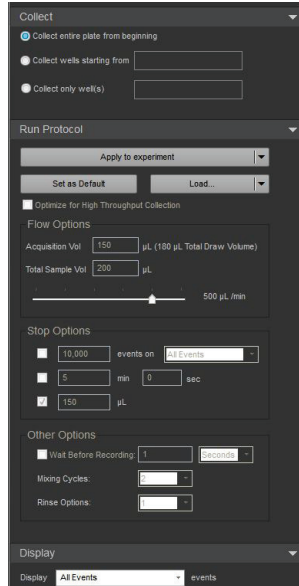
- Select "Collect entire plate from beginning"
- Acquisition volume: 150 µL
- Total sample volume: 200 µL
- Flow rate: 500 µL/min
- Stop options: 150 µL
- Mixing cycles: 2
- Rinse options: 1
- Display: all events

6.4 Add 200 µL of well-mixed labeled sample (from step 2.11) to each well of the first 96-well plate.

6.5 Place the 96-well plate in the Attune NxT Autosampler and select "Run Plate".

6.6 When acquisition of the first plate is complete, repeat steps 6.4 and 6.5 with the second plate.

6.7 Adjust the gating using the FMO controls to analyze data (Figure 4).

Figure 4. Representative data and gating example. Plot A is gated on all events with CD45 vs. SSC showing all three WBC populations, distinct from debris. A gate is drawn around the CD45+ lymphocyte population, used to gate plots C, D, and E. Plot B of the time parameter vs. SSC is gated on all events, demonstrating steady and consistent data acquisition over the entire sample collection. Plot C of CD3 vs. CD4 shows expected patterns and identifies CD45+ CD3+ CD4+ T helper cells with the percent positive of the parent lymphocyte gate displayed in each quadrant. Plot D of CD3 vs. CD8 shows expected patterns and identifies CD45+ CD3+ CD8bright+ T suppressor cells as distinct from CD45+ CD3− CD8dim+ cells, with the percent positive of the parent gate displayed in each quadrant. Plot E shows mutually exclusive T cell populations of CD4 vs. CD8 where no co-positivity is demonstrated, with the percent positive of the parent gate displayed in each quadrant.
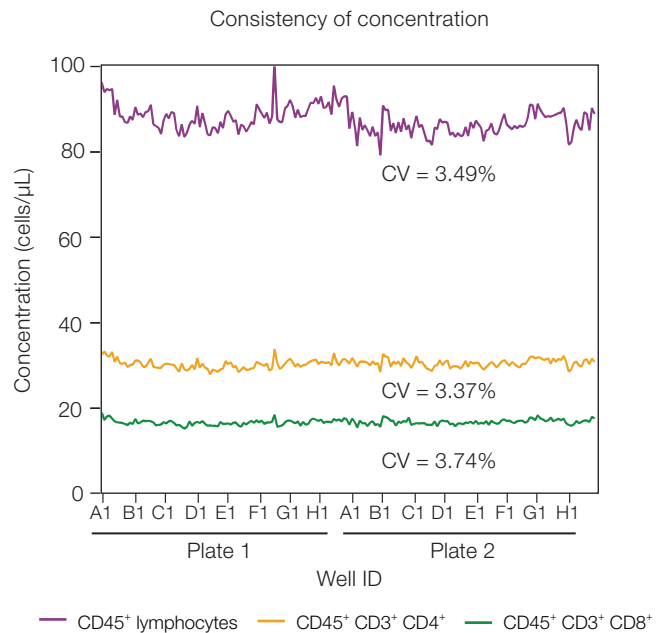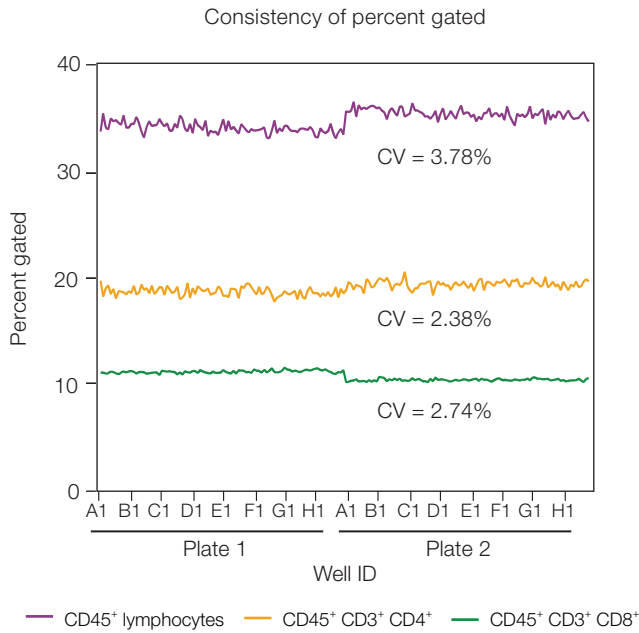
Consistency of percent gated

Figure 5. Display of the percent positive of three gated cell populations over two 96-well plates. These statistics are displayed for each well of both 96-well plates. Coefficients of variation for each measurement were less than 4%.

Consistency of concentration

Figure 6. Display of the concentration statistic of three gated populations over two 96-well plates. The coefficient of variation is shown for each.
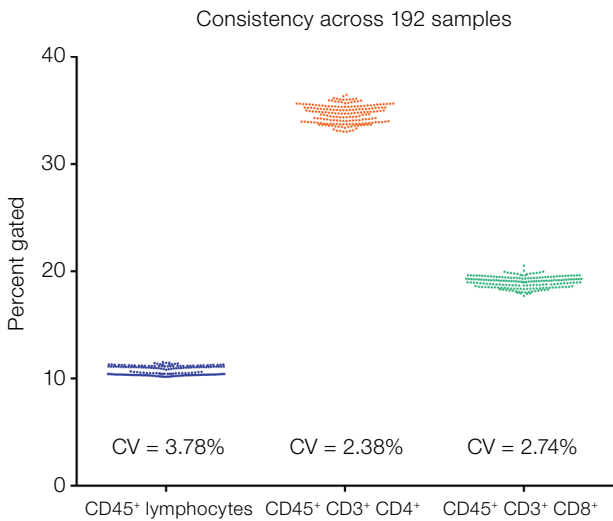

Consistency across 192 samples

Figure 7. Consistency of all 192 wells for analysis of percent positive of parent gate of the three identified populations.

Consistency across 192 samples
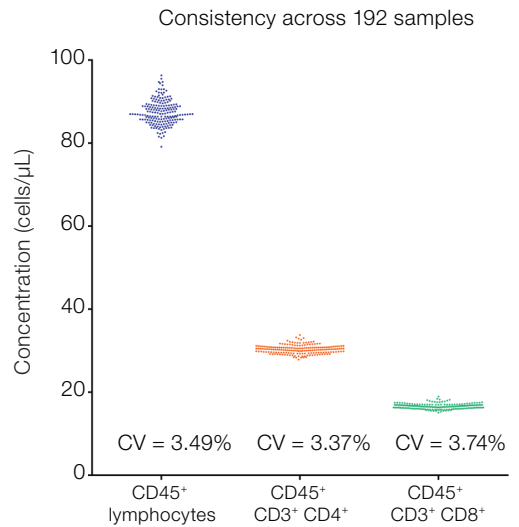
Figure 8. Consistency of all 192 wells for analysis of concentration of the three identified populations.

Results

The combination of fluorophores and antibodies in this experiment were chosen specifically to reduce the amount of compensation. The four fluorophores are each detected by a different laser. Compensation values were determined using automatic compensation and found to be minimal (Figure 2). The FMO controls were useful to ensure optimal placement of gates. Acquisition of each 96-well plate was completed in approximately 43 min. Consistent results were observed across wells on a 96-well plate and between 96-well plates, with coefficients of variation observed below 4% for each population of cells when analyzing percent parent and concentration in cells/µL.

Figures 5–8 show the consistency across each 96-well plate and for all 192 replicate samples for percent positive and concentration of the three cell populations. The CD45+ lymphocytes are a subset of all events, including all debris events. The CD45+ CD3+ CD4+ and CD45+ CD3+ CD8+ populations are gated on the CD45+ lymphocyte gate, and thus represent T cell subsets.

Another method for evaluating consistency is the Heat Map analysis function in Attune NxT Software. The Heat Map view shows a virtual plate layout that represents the wells available on the 96-well plate. The Heat Map Setup panel allows selection of the statistic, gate, and parameter for visualizing the data from the experiment, and definition of the display mode and transition values to analyze the data at a glance. The Heat Map view may be used for analyzing tube-based data in addition to plate-based data. Figure 9 illustrates this visual display, using the concentration (in cells/µL) of the CD45+ CD3+ CD4+ cell population. The selected parameter value for each well is displayed in the corresponding graphic well, and the color is selected by the location of the value on the color transition display.
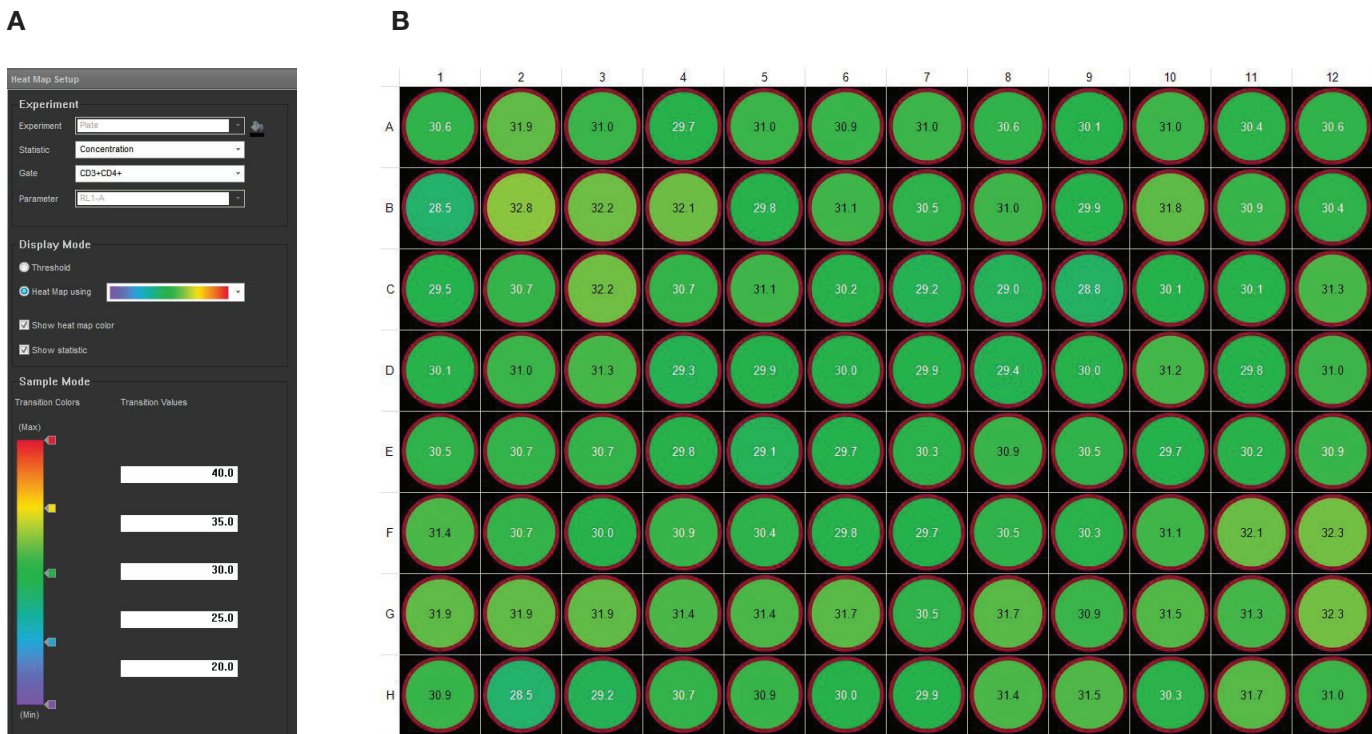
A

B

Figure 9. Heat Map view and analysis. (A) Heat Map Setup panel and **(B)** display using settings as defined in (A). The transition values and colors are used to display the results in an easy-to-view format.

invitrogen

Conclusions

Automation helps maximize reproducibility between experiments and reduces inter-assay variability and inter-operator variability. With low CVs across and among plates, the Attune NxT Flow Cytometer with Autosampler offers a reliable and high-throughput approach for multiparametric cell screening from 96- and 384-well plates, allowing analysis from smaller sample quantities to maximize efficient use of limited numbers of cells.

This combination of higher-throughput with higher-content flow cytometric analysis of as many as 16 parameters for each cell, with acquisition speeds of up to 1 mL/min, provides a powerful screening platform for drug discovery and systems biology experiments. For further information, read our application note about recommendations for accurate concentration measurements on the Attune NxT Flow Cytometer [6].

References

1. Attune NxT Software user guide, Pub. No. 100024236, Rev. F.
2. Attune NxT Flow Cytometer user guide, Pub. No. 100024235, Rev. C.0.
3. Attune NxT Autosampler user guide, Pub. No. 100032905, Rev. A.0.
4. Countess II Automated Cell Counter user guide, Pub. No. MAN0014293, Rev. B.0.
5. Roederer M (2002) Compensation in flow cytometry. *Curr Protoc Cytom* 22:1.14.1–1.14.20.
6. Application note: Recommendations for accurate concentration measurements on the Attune NxT Flow Cytometer, Pub. No. COL13402.