

# Validation of Th17 Cell Differentiation from Peripheral Blood CD4+ Cells through Assessment of mRNA Expression and Cytokine Secretion using Microplate Reading and Cellular Imaging

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## Introduction

CD4+ T cells signal and regulate an immune response to pathogens after interacting with an antigen-MHC (major histocompatibility complex). This activation causes the cells to differentiate into distinct phenotypic and functional effectors, collectively referred to as T helper cells (Th), depending on specific cytokine signaling and transcription factors and epigenetic modifications. One such differentiated T cell subset are the pro-inflammatory T helper 17 (Th17) cells. These cells can be beneficial to the host during infection, as they amplify ongoing inflammation by inducing expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). However, uncontrolled activation of Th17 cells are associated with multiple inflammatory and autoimmune disorders including arthritis, primary Sjögren's syndrome (pSS), multiple sclerosis (MS), and cancer. Naïve CD4+ T cells that are stimulated into Th17 differentiation are uniquely characterized by production of the pro-inflammatory cytokine, IL-17. Research has shown that targeting the IL-17 pathway has attenuated disease severity in preclinical models of autoimmune diseases, which has caused a growing interest in their use as a potential therapeutic target.

The process of attaining viable Th17 cells typically includes multiple steps. CD4+ T cells are isolated from peripheral blood mononuclear cells (PBMCs) and further selected against CD4. Purified cells are then differentiated into Th17 cells using a cocktail of specific antibodies and cytokines. The entire procedure to create and confirm this differentiation can be labor and time intensive.

Here we demonstrate a validated, robust method to differentiate cryopreserved peripheral blood CD4+ T cells into functional Th17 cells using a bead-based activation technology. A novel cell imaging multi-mode reader is used to image phenotypic differences between cells exposed to the antibody/cytokine cocktail and negative control cells as differentiation proceeds. Creation of fully functioning Th17 cells was then confirmed by assessing IL-17F mRNA levels using a fluorescence RNA *in situ* hybridization (RNA FISH) assay, in addition to IL-17 secretion using a homogeneous, bead-based immunoassay technology. The reader previously described performed all brightfield and fluorescence imaging steps, as well as laser-based excitation for the secretion immunoassay. The combination provides a comprehensive solution for the creation and validation of this important class of helper CD4+ T cells.

## BioTek Instrumentation



**Cytation™ 5 Cell Imaging Multi-Mode Reader:** Cytation 5 is a modular multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation 5 includes filter- and monochromator-based microplate reading, and offers laser-based excitation for Alpha assays. The microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C (37 ± 0.2 °C), CO<sub>2</sub>/O<sub>2</sub> gas control and dual injectors for kinetic assays. Shaking and Gen5™ Data Analysis Software are also standard. The instrument was used for image-based phenotypic differentiation monitoring, in addition to microplate reader- and image-based validation of Th17 helper T cell creation.

## Materials

**Miltenyi Th17 Cell Activation/Expansion Components:** The T Cell Activation/Expansion Kit was developed to activate and expand human T cells. The base kit consists of Anti-Biotin MACS bead™ Particles and biotinylated antibodies against human CD2, CD3, and CD28. The optimized protocol for Th17 cell differentiation consists of the following components which were purchased from Miltenyi Biotec, Inc. (San Diego, CA): T Cell Activation/Expansion Kit, Human (Catalog No. 130-091-441), TexMACS™ Medium, Research Grade (Catalog No. 130-097-196), Human IL-1 $\beta$ , Premium Grade (Catalog No. 130-093-897), Human IL-6, Premium Grade (Catalog No. 130-095-352), Human IL-23, Research Grade (Catalog No. 130-095-757), Human TGF- $\beta$ 1, Premium Grade (Catalog No. 130-095-067), anti-IFN- $\gamma$ , Pure-Functional Grade, Human (Catalog No. 130-095-743), and anti-IL-4, Pure, Human (Catalog No. 130-108-049).

**Microplates:** Falcon® 24 well polystyrene clear flat bottom not treated cell culture plates (Catalog No. 351147) and Falcon 96 well clear round bottom not treated microplates (Catalog No. 351177) were donated by Corning Life Sciences (Corning, NY). AlphaPlate-384, Light Gray, untreated (Catalog No. 6005350) was provided by PerkinElmer, Inc., (Waltham, MA).

**Cells:** Peripheral Blood CD4+ T Cells (Catalog No. 2W-200) were purchased from Lonza Group, Ltd. (Basel, Switzerland).

**Validation Assay Chemistries:** AlphaLISA® IL-17 Immunoassay Research Kit (Catalog No. AL219C) was donated by PerkinElmer, Inc. QuantiGene® ViewRNA™ ISH Cell Assay Kit (Catalog No. QVC0001), ViewRNA Probe Human IL17F (RUO) (Catalog No. VA1-10122-01) and ViewRNA Probe Human ACTB (RUO) (Catalog No. VA6-10506-01) were donated by Affymetrix, Inc., (Santa Clara, CA).

## Methods

**Anti-Biotin MACS bead Particle Loading:** 100  $\mu$ L each of CD2-Biotin, CD3-Biotin, and CD28-Biotin antibodies (100  $\mu$ g/mL) were added to a 2 mL tube and mixed. 500  $\mu$ L of anti-biotin MACS bead particles (1.0x10<sup>8</sup> total particles) were then added to the antibody mix. Finally, 200  $\mu$ L of PBS pH 7.2, supplemented with 0.5% human serum albumin and 2 mM EDTA, was added to the tube and mixed. The tube was incubated at 4 °C for two hours with gentle rotation. Following incubation, the tube of loaded particles was kept at 4 °C until the beginning of the differentiation procedure.

**CD4+ Cell Differentiation Procedure:** Cryopreserved CD4+ cells were thawed, added to TexMACS Medium, centrifuged at 1000 RPM for 8 minutes, and then resuspended at a concentration of 1.1x10<sup>6</sup> cells/mL. An aliquot of MACS bead particles sufficient for the experiment was removed from the stored beads, added to 200  $\mu$ L of medium, and centrifuged at 300xg for five minutes. The beads were then resuspended at a concentration of 5.0x10<sup>5</sup> beads per 100  $\mu$ L of medium. Cells, beads, cytokines, and antibodies were then added together in the final concentrations below:

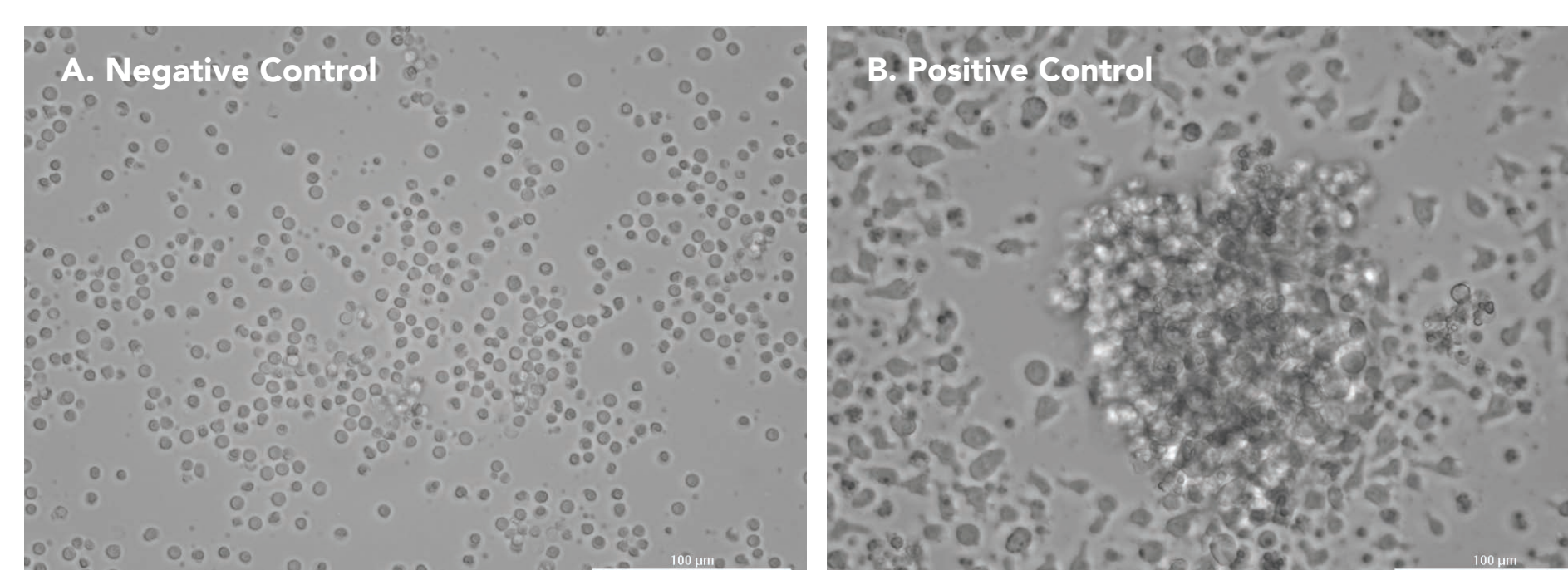
Differentiation Component	Concentration
CD4+ Cells	1.0x10 <sup>6</sup> /mL
Loaded MACS bead Particles	5.0x10 <sup>5</sup> /mL
IL-1 $\beta$	20 ng/mL
IL-6	30 ng/mL
IL-23	30 ng/mL
TGF- $\beta$ 1	2.25 ng/mL
Anti-IFN- $\gamma$ Antibody	1 $\mu$ g/mL
Anti-IL-4 Antibody	2.5 $\mu$ g/mL

Four 1 mL aliquots and four 200  $\mu$ L aliquots were added to separate wells of a 24-well and 96-well plate, respectively. CD4+ cells, in the absence of loaded particles, were also added to the same plates as a negative control. Plates were placed in a 37 °C/5% CO<sub>2</sub> incubator for 7 days without medium exchange.

**Validation of Th17 Cell Creation:** Following incubation, medium aliquots were removed for cytokine secretion determination with the AlphaLISA IL-17 assay. Cells were then resuspended and transferred to an imaging plate to analyze mRNA expression using the QuantiGene ViewRNA ISH Cell Assay.

## Phenotypic Monitoring of Cell Differentiation

Image-based monitoring of Th17 cell differentiation was performed during the incubation period. 24- and 96-well plates were placed into the Cytation 5, with the imaging chamber previously set to 37 °C/5% CO<sub>2</sub>. Brightfield images were captured to assess potential phenotypic differences taking place in the cells and culture.

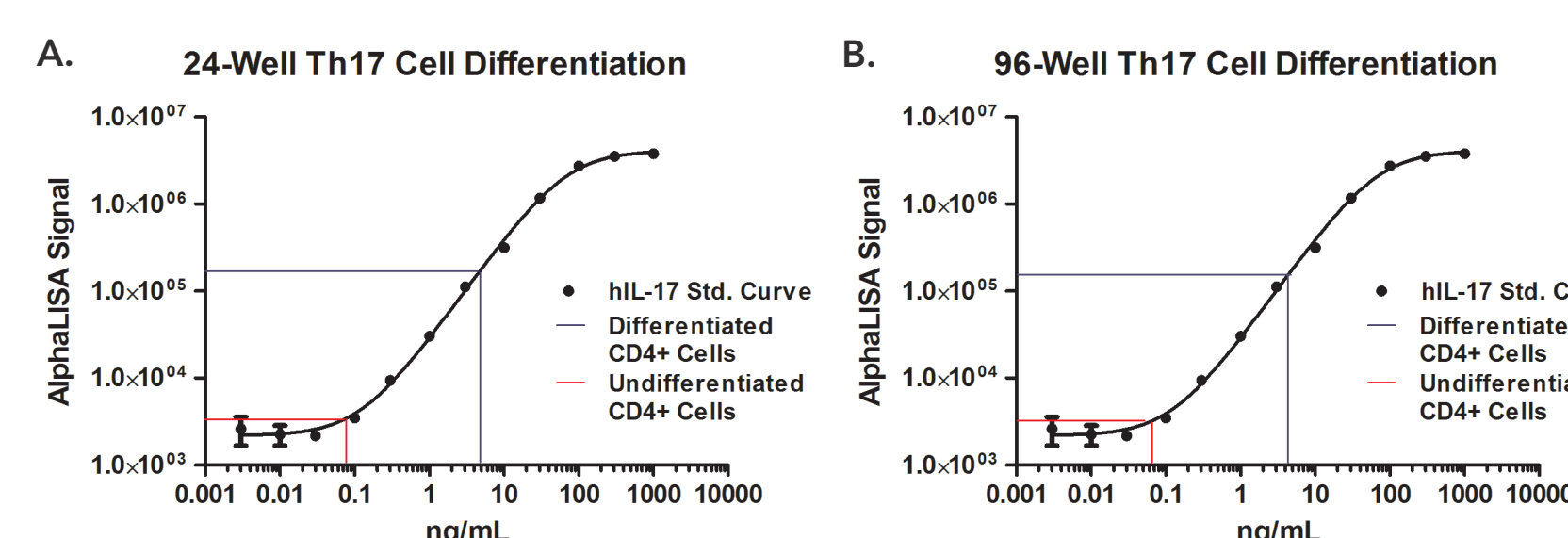


**Figure 1. Brightfield Images of Differentiating CD4+ Cells.** Images captured from 24-well differentiation plate of (A) negative and (B) positive control wells using 20x objective.

Anti-Biotin MACS bead Particles loaded with biotinylated antibodies are used to mimic antigen-presenting cells and activate resting T cells. Cells are attracted to the bound antibodies, causing creation of the Th17 lineage in the presence of appropriate cytokines and additional antibodies. Therefore a positive indication of differentiation is visible cell clumping around the bead particles. Activated cells also demonstrate increased signs of proliferation. These phenotypes are seen in the images captured from 24-well positive control wells and 96-well positive control wells. Organization around bead particles and actively proliferating cells are visualized, which is absent from negative control wells (Figure 1).

## Validation of hIL-17 Secretion

At the conclusion of the seven day incubation, two 5  $\mu$ L aliquots of cell medium were removed from the four positive control wells in the 24- and 96-well differentiation plates and transferred to a light gray 384-well AlphaPlate, in addition to duplicate aliquots from negative control wells. A titration of hIL17 analyte was created and transferred to the same assay plate. The remaining steps of the AlphaLISA assay were then performed according to the vendor protocol.

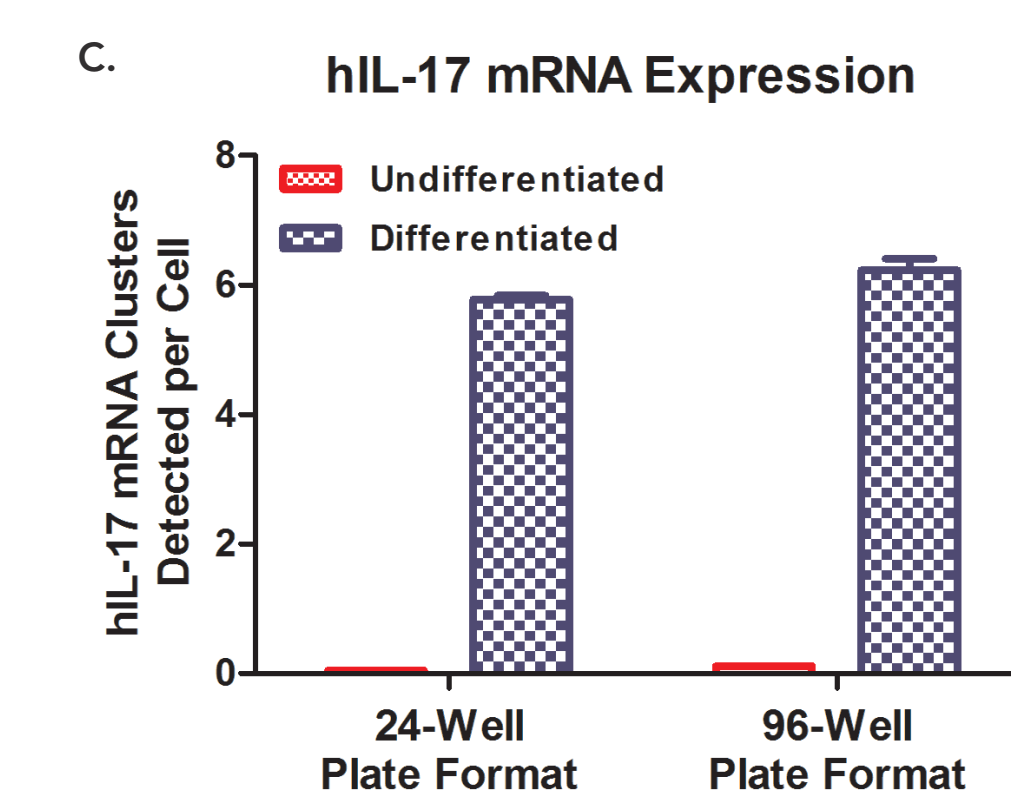
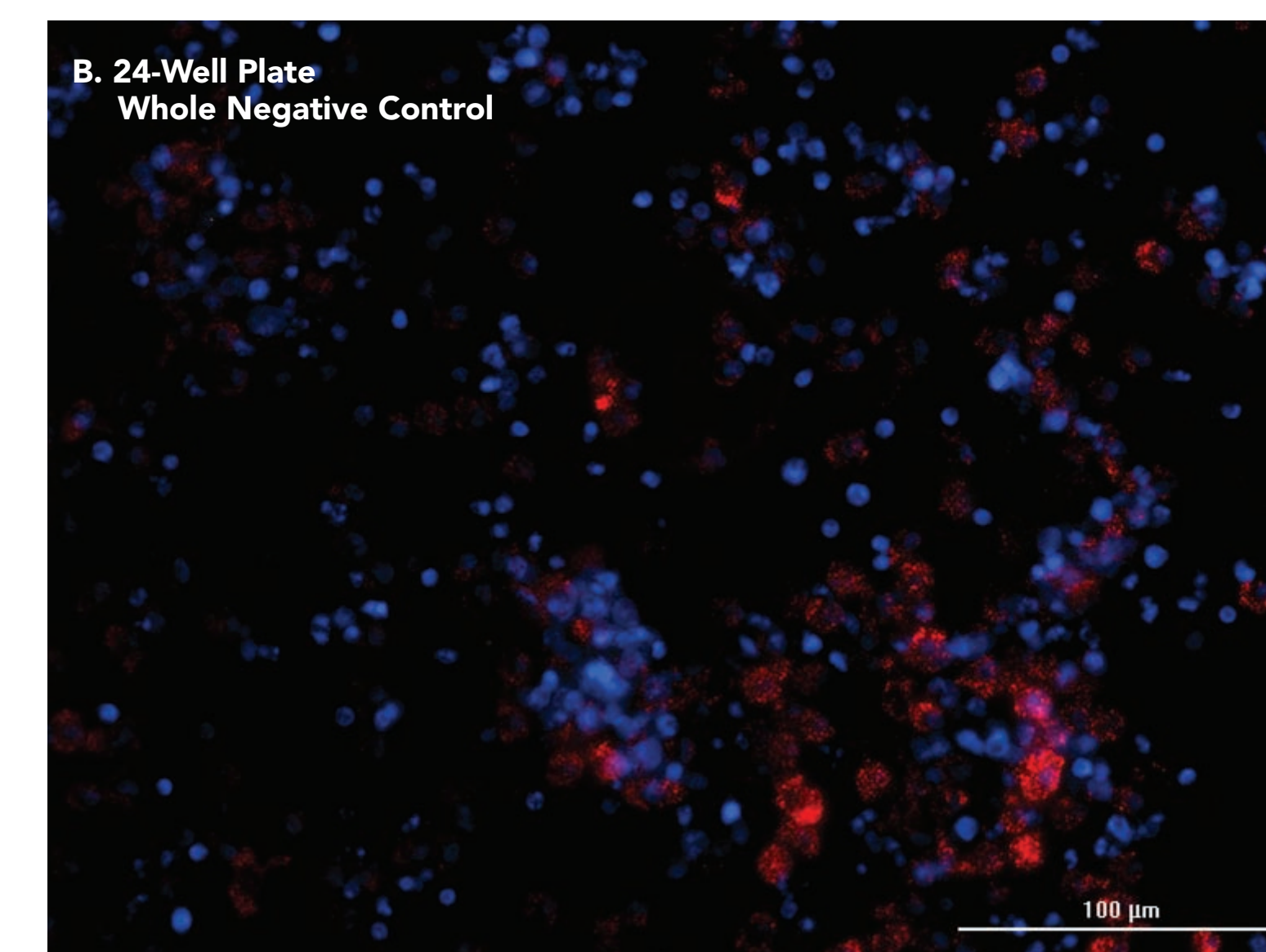
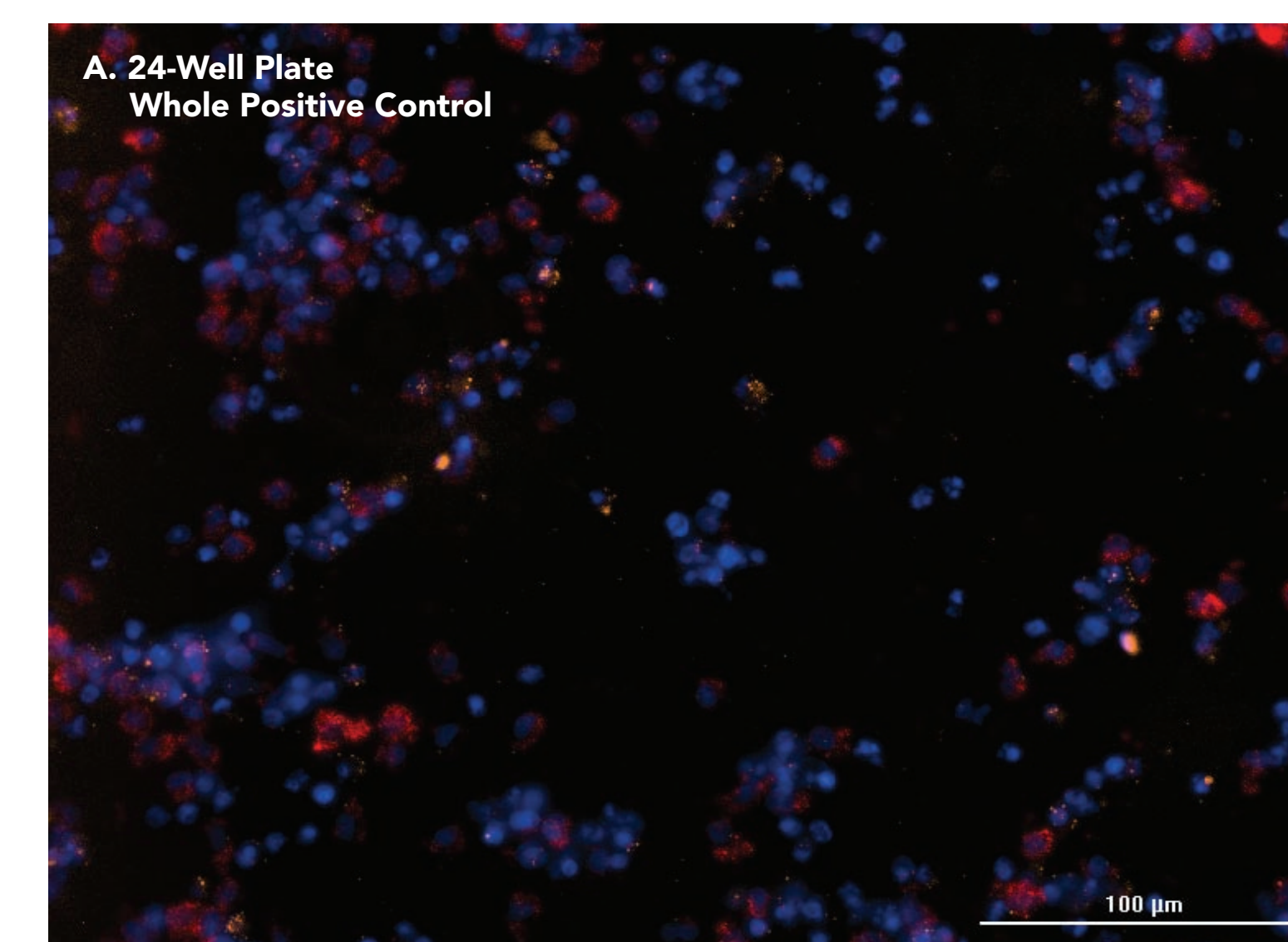


**Figure 2. hIL-17 Secretion Determination.** Average secreted cytokine concentrations from 8 replicate measurements for (A) 24- and (B) 96-well differentiation plates.

It was found that IL-17 supernatant concentrations were 4.8 ± 0.6 ng/mL in 96-well plates and 4.5 ± 0.7 ng/mL in 24-well plates. These concentrations represent a greater than 50-fold increase in IL-17 relative to negative controls. This validates that the bead-based differentiation process activates CD4+ cells into IL-17 secreting cells.

## ISH-Based Analysis of IL-17F mRNA Expression

Validation of Th17 cell creation was also performed at the RNA level through incorporation of a fluorescence RNA *in situ* hybridization technique. Cells were removed from the wells of the 24- and 96-well differentiation plates, fixed, and added to a poly-L-lysine imaging plate. The plate was incubated at 50 °C for 30 minutes to dry the cells and increase adherence to the plate. The remainder of the hybridization procedure was then carried out. Fluorescence imaging was then completed to ascertain levels of IL-17F mRNA expression.



**Figure 3. hIL-17F mRNA Assessment.** (A) Whole positive control image captured using a 20x objective from cells differentiated in 24-well format. (B) Whole negative control images from undifferentiated cells taken from 24-well plates. Red: ACTB mRNA expression imaged using Cy5 imaging channel; Orange: IL-17F mRNA expression imaged using RFP imaging channel; Blue: DAPI labeled nuclei imaged using DAPI imaging channel. (C) Graph of differentiated and undifferentiated mRNA expression per imaged cell.

Images from wells containing differentiated cells exhibit a higher level of visible IL-17F mRNA expression compared to wells containing undifferentiated cells (Figure 3A-C). This is further confirmed through cellular analysis performed on each test well, where a greater than 50-fold increase in mRNA expression is seen from positive control wells.

## Conclusions

- The bead-based protocol using MACS bead particles creates activated Th17 immune cells suitable for downstream applications.
- Cryopreserved CD4+ cells from Lonza simplify differentiation by removing isolation and propagation steps from the final procedure.
- The AlphaLISA IL-17 assay provides an easy-to-use, responsive method to determine analyte secretion from positively differentiated cells.
- ViewRNA fluorescent ISH cell assays allow for sensitive analyte mRNA expression detection.
- The Cytation 5 Cell Imaging Multi-Mode Reader provides laser excitation, in addition to PMT and CCD-based detection to enable monitoring and validation of differentiation in a single instrument.
- The combination of differentiation procedure, cells, assessment methods and instrumentation provide a simplified, robust process for the creation and validation of activated immune cells.

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