

Titrating Antibodies on Human PBMC

When staining cells with antibodies, background non-specific staining needs to be minimized. We titrate our antibodies to determine the optimal antibody concentration yielding the greatest signal for the positive population and the lowest background signal for the negative population. We generally start our titrations at 2x the antibody manufacturer's suggested volume or concentration necessary to stain 10^6 cells; next we perform four serial 1:1 dilutions from this starting point.

In this exercise, four antibodies stain ficolled human peripheral blood mononuclear cells (PBMC) cell surface antigens. These antibodies are Biolegend Brilliant Violet 605 Anti-Human CD4 (clone OKT4, catalog # 317438), Beckman Coulter PE-Cy5.5 Anti-Human CD3 (clone UCHT1, catalog # A66327), Beckman Coulter PE-Cy7 Anti-Human CD14 (clone RMO52, catalog # A22331), Beckman Coulter FITC Anti-Human CD16 (clone 3G8, catalog # IM0814U).

Procedure:

1. For all four antibodies, we will start the titrations at double the manufacturers' recommended volume of antibody necessary to stain 10^6 cells.

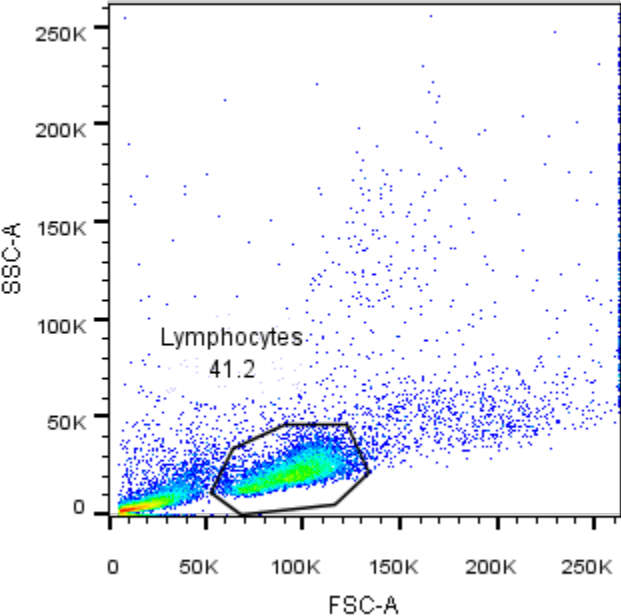
The table below details the titration scheme:

| Antibody | Titration 1 | Titration 2 | Titration 3 | Titration 4 | Titration 5 |
|--------------|-------------|-------------|-------------|-------------|--------------|
| CD4 BV605 | 5 μ l | 2 μ l | 1 μ l | 0.5 μ l | 0.1 μ l |
| CD3 PE-Cy5.5 | 20 μ l | 10 μ l | 5 μ l | 2.5 μ l | 1.25 μ l |
| CD14 PE-Cy7 | 20 μ l | 10 μ l | 5 μ l | 2.5 μ l | 1.25 μ l |
| CD16 FITC | 40 μ l | 20 μ l | 10 μ l | 5 μ l | 2.5 μ l |

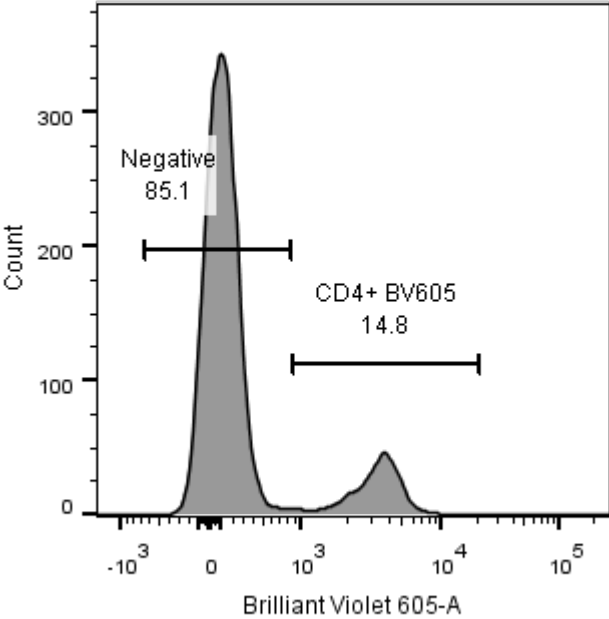
Manufacturers' recommended volume for staining 10^6 cells (we did not start at 2x for CD4 BV605 because we have titered this Ab and we know it's very bright).

2. Add 1×10^6 PBMC in 90 μ l in wash buffer (1% BSA or FCS in PBS). Add 10 μ l of 50 mg/ml normal mouse serum (Jackson ImmunoResearch).
3. Incubate at room temperature for 30 minutes.
4. Wash with 2 ml of wash buffer. Spin down at 300 x g. Repeat.
5. Add 250 μ l wash buffer.
6. Run the cells on the LSR II.

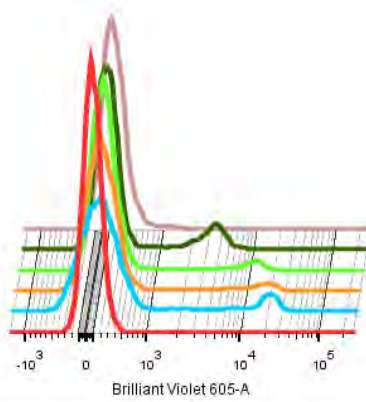
Results for Biolegend Brilliant Violet 605 Anti-Human CD4 (clone OKT4, catalog # 317438)
Gate on the lymphocytes:



Gate on the Positive and Negative cell populations:

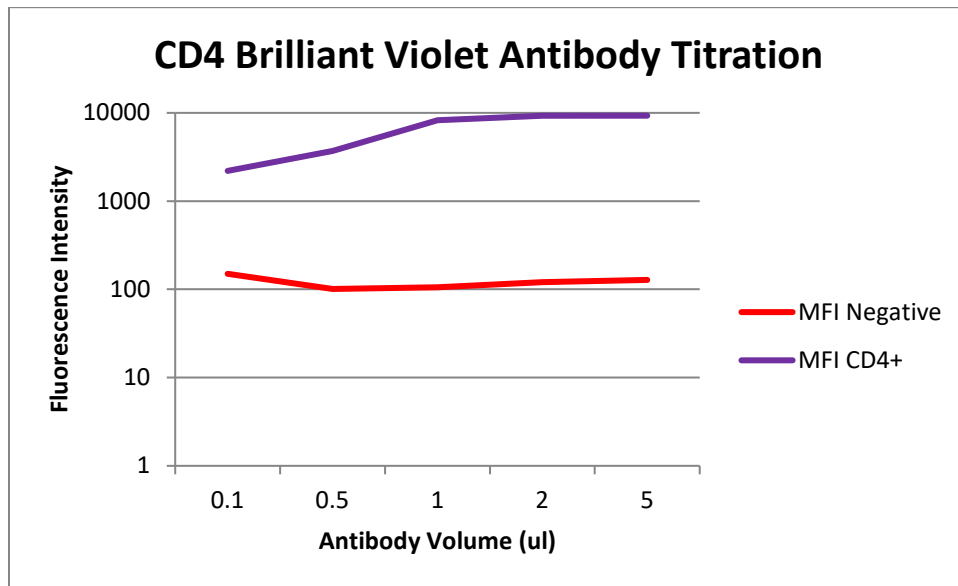


Overlay all antibody concentrations:



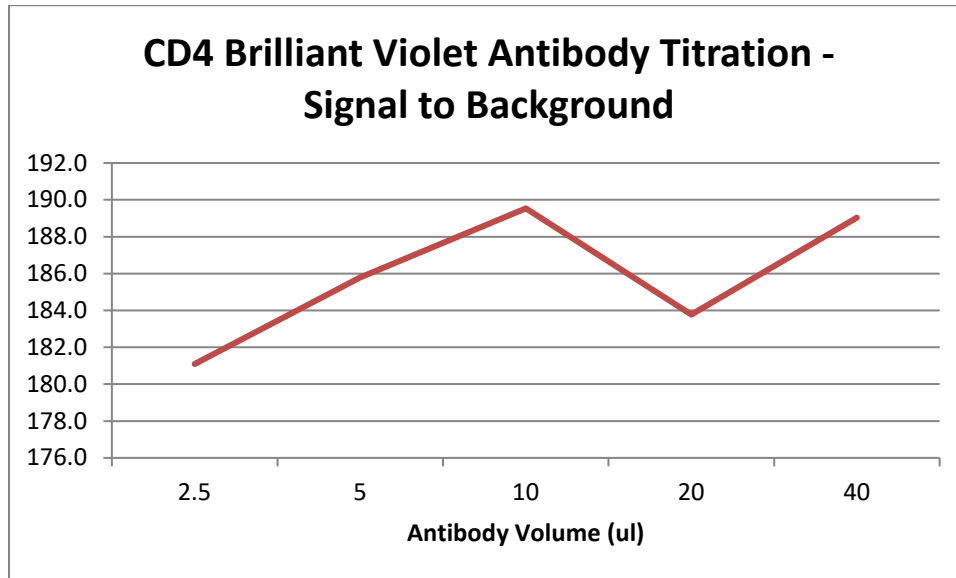
| | Sample Name | Subset Name | Count |
|---|------------------------------------|-------------|-------|
| □ | Specimen_001_Unstained Cells.fcs | Lymphocytes | 8651 |
| □ | Specimen_001_CD4 BV 605 5 ul.fcs | Lymphocytes | 8395 |
| □ | Specimen_001_CD4 BV 605 2 ul.fcs | Lymphocytes | 8358 |
| □ | Specimen_001_CD4 BV 605 1 ul.fcs | Lymphocytes | 8429 |
| □ | Specimen_001_CD4 BV 605 0.5 ul.fcs | Lymphocytes | 8477 |
| □ | Specimen_001_CD4 BV 605 0.1 ul.fcs | Lymphocytes | 8713 |

Plot the Mean Fluorescence Intensity for the Antibody Positive and Negative populations:



| CD4 Brilliant Violet 605 Antibody Titration | | |
|---|--------------|----------|
| Antibody Volume (μl) | MFI Negative | MFI CD4+ |
| 0.1 | 150 | 2201 |
| 0.5 | 101 | 3696 |
| 1 | 106 | 8258 |
| 2 | 121 | 9288 |
| 5 | 128 | 9293 |

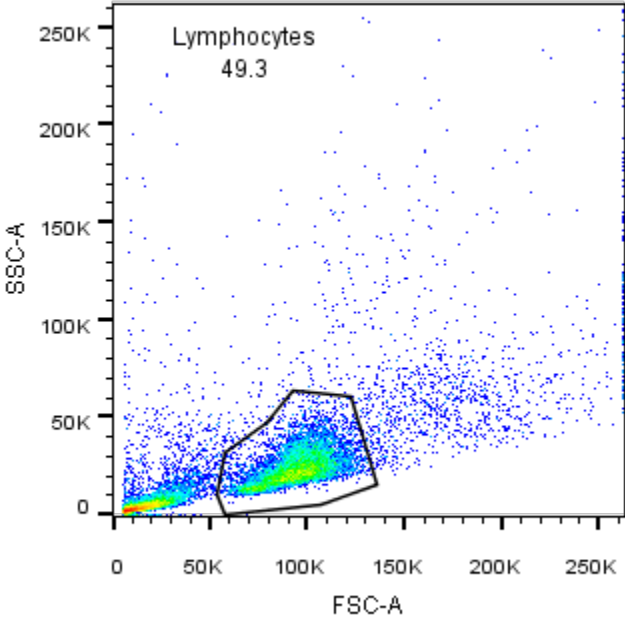
Calculate the Signal (positive Ab staining) vs the Background (negative staining):



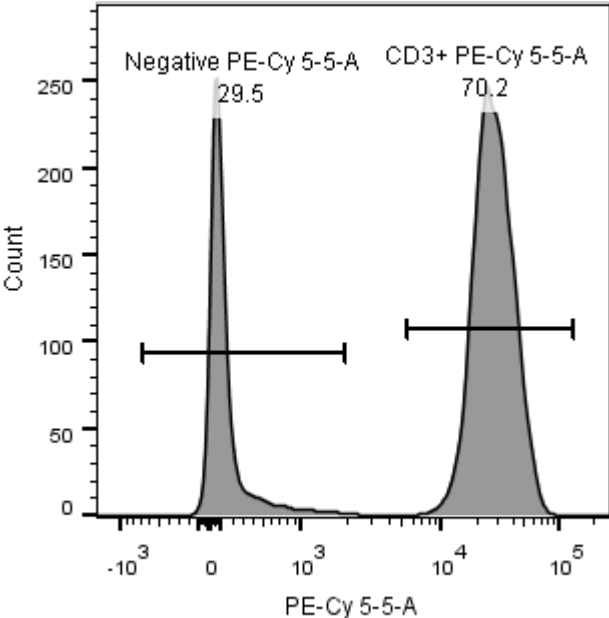
| Antibody Volume (μ l) | Signal To Background |
|----------------------------|----------------------|
| 0.1 | 14.7 |
| 0.5 | 36.6 |
| 1 | 77.9 |
| 2 | 76.9 |
| 5 | 72.6 |

For CD4 Brilliant Violet 605, the best antibody volume per test to use is 1 μ l because it has the greatest signal to background ratio. Biologend recommends 5 μ l/test. Using 1 μ l/test is a significant savings compared to 5 μ l/test.

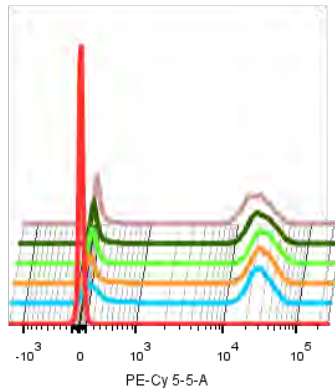
Results for Beckman Coulter PE-Cy5.5 Anti-Human CD3 (clone UCHT1, catalog # A66327)
Gate on the Lymphocytes:



Gate on the Positive and Negative cell populations:

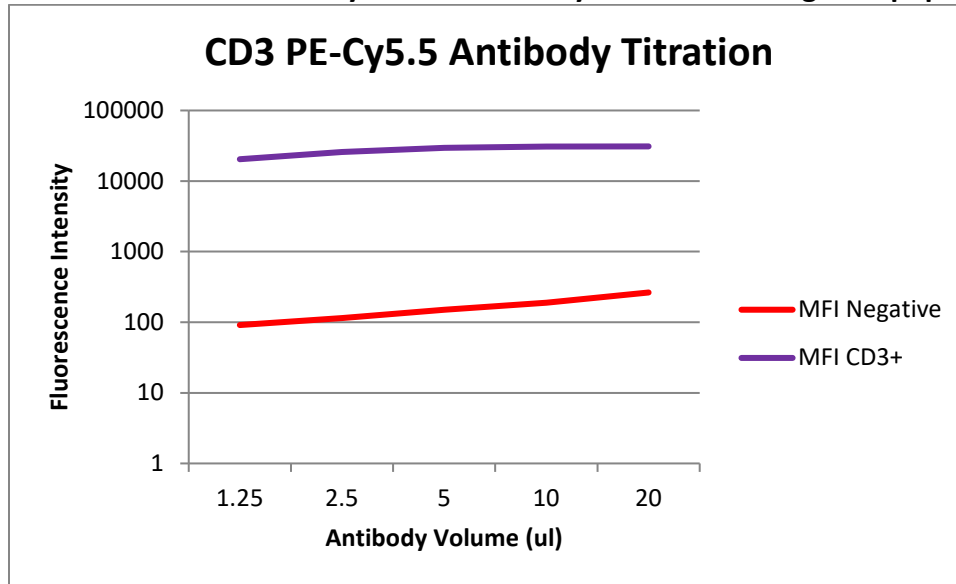


Overlay all antibody concentrations:



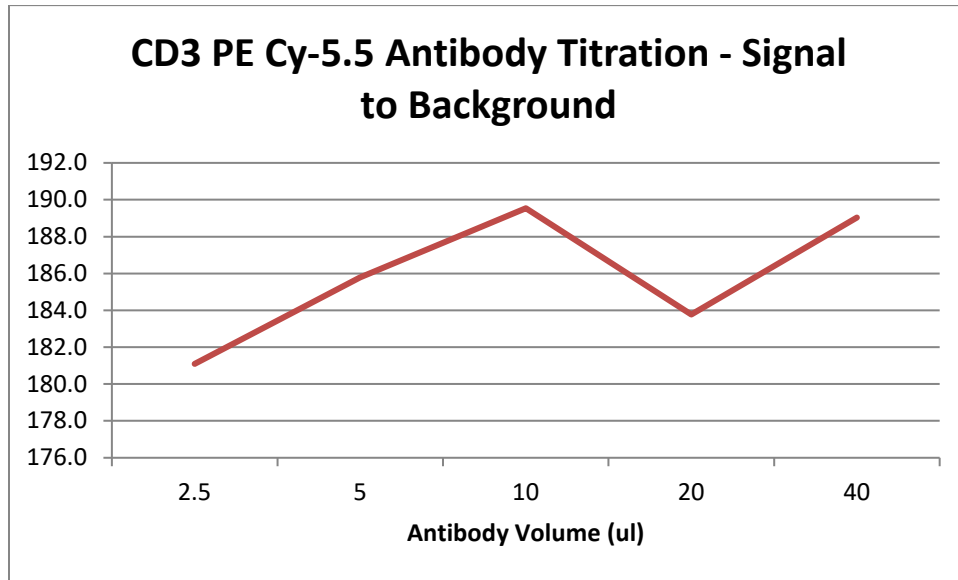
| | Sample Name | Subset Name | Count |
|---|---------------------------------------|-------------|-------|
| 1 | Specimen_001_Unstained Cells.fcs | Lymphocytes | 8851 |
| 2 | Specimen_001_CD3 PE-Cy5 5 20 ul.fcs | Lymphocytes | 9143 |
| 3 | Specimen_001_CD3 PE-Cy5 5 10 ul.fcs | Lymphocytes | 9044 |
| 4 | Specimen_001_CD3 PE-Cy5 5 5 ul.fcs | Lymphocytes | 8902 |
| 5 | Specimen_001_CD3 PE-Cy5 5 2.5 ul.fcs | Lymphocytes | 8874 |
| 6 | Specimen_001_CD3 PE-Cy5 5 1.25 ul.fcs | Lymphocytes | 8866 |

Plot the Mean Fluorescence Intensity for the Antibody Positive and Negative populations:



| CD3 PE-Cy5.5 Antibody Titration | | |
|---------------------------------|--------------|----------|
| Antibody Volume (μl) | MFI Negative | MFI CD3+ |
| 1.25 | 91.3 | 20396 |
| 2.5 | 114 | 25749 |
| 5 | 150 | 29570 |
| 10 | 189 | 30798 |
| 20 | 264 | 30970 |

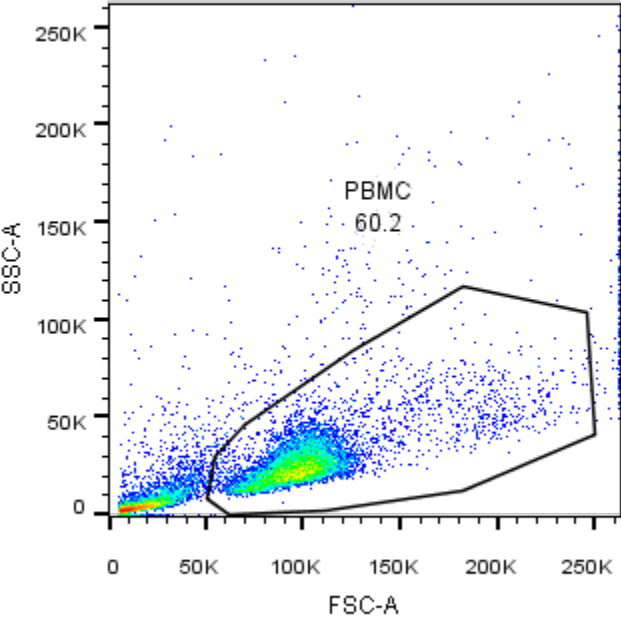
Calculate the Signal (positive Ab staining) vs the Background (negative staining):



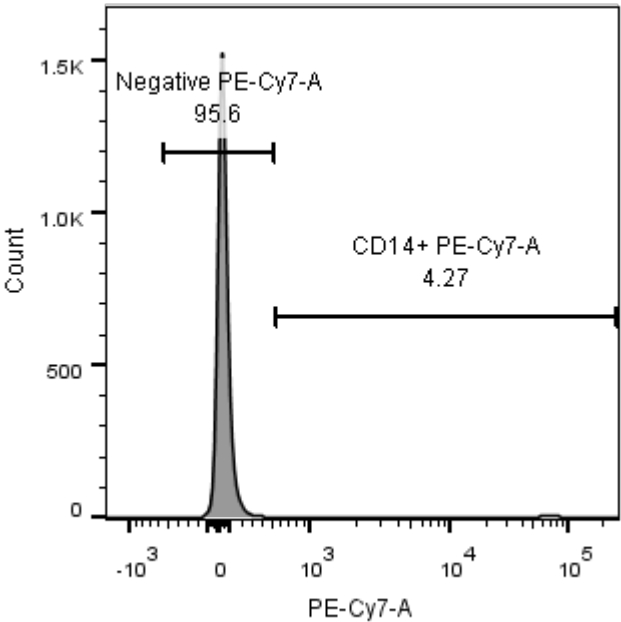
| Antibody Volume (μ l) | Signal To Background |
|----------------------------|----------------------|
| 1.25 | 223.4 |
| 2.5 | 225.9 |
| 5 | 197.1 |
| 10 | 163 |
| 20 | 117 |

For CD3 PE-Cy5.5, the best antibody volume per test to use is 2.5 μ l because it has the greatest signal to background ratio. Beckman Coulter recommends 10 μ l/test.

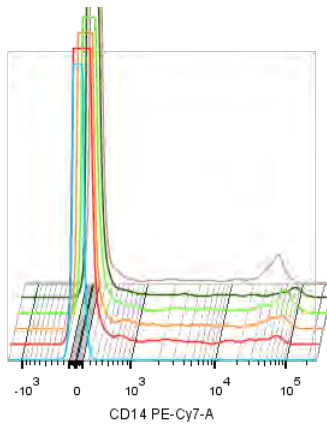
**Beckman Coulter PE-Cy7 Anti-Human CD14 (clone RMO52, catalog # A22331)
Gate on the Mononuclear cells:**



Gate on the Positive and Negative cell populations:

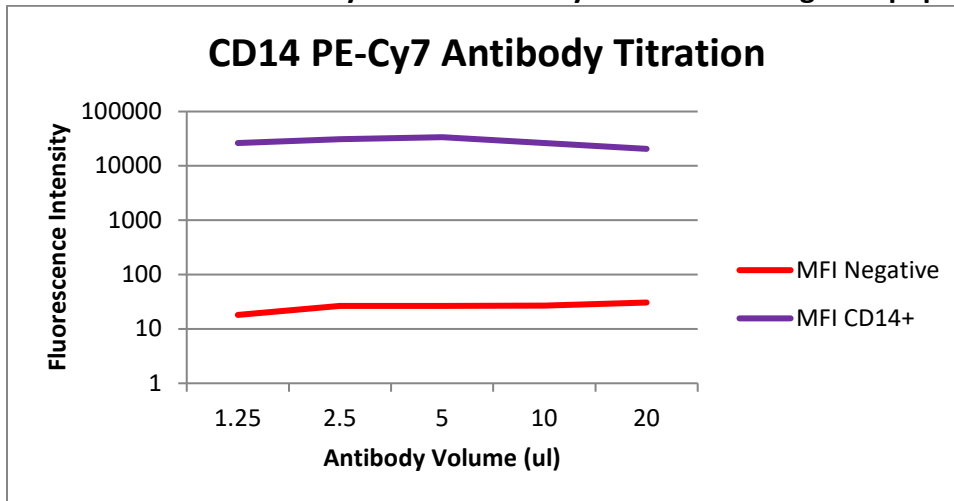


Overlay all antibody concentrations:



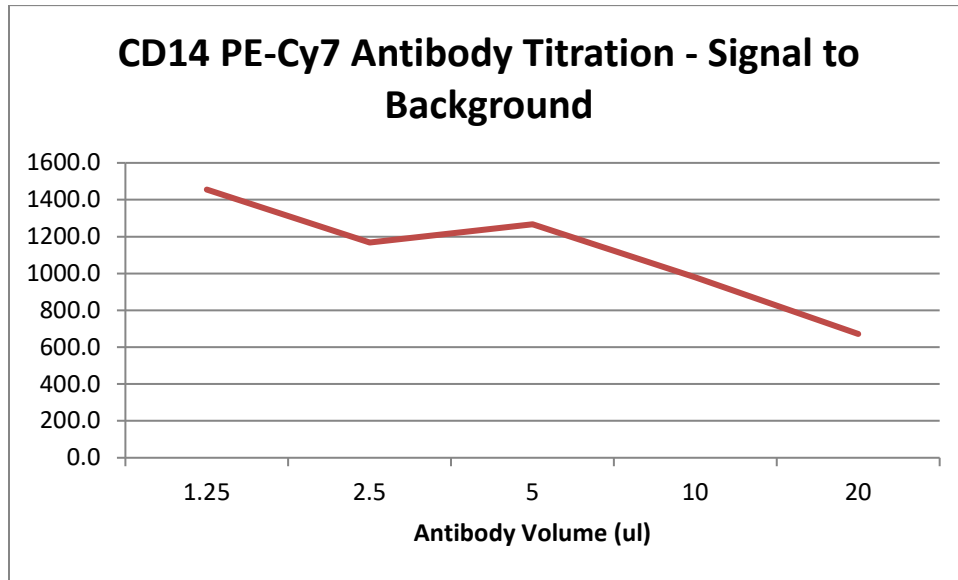
| | Sample Name | Subset Name | Count |
|--|--------------------------------------|-------------|-------|
| | Specimen_001_Unstained Cells.fcs | PBMC | 9850 |
| | Specimen_001_CD14 PE-Cy7 20 ul.fcs | PBMC | 9872 |
| | Specimen_001_CD14 PE-Cy7 10 ul.fcs | PBMC | 9850 |
| | Specimen_001_CD14 PE-Cy7 5 ul.fcs | PBMC | 9862 |
| | Specimen_001_CD14 PE-Cy7 2.5 ul.fcs | PBMC | 9826 |
| | Specimen_001_CD14 PE-Cy7 1.25 ul.fcs | PBMC | 9801 |

Plot the Mean Fluorescence Intensity for the Antibody Positive and Negative populations:



| CD14 PE-Cy7 Antibody Titration | | |
|--------------------------------|--------------|-----------|
| Antibody Volume (ul) | MFI Negative | MFI CD14+ |
| 1.25 | 18.1 | 26339 |
| 2.5 | 26.6 | 31082 |
| 5 | 26.6 | 33711 |
| 10 | 27 | 26420 |
| 20 | 30.6 | 20557 |

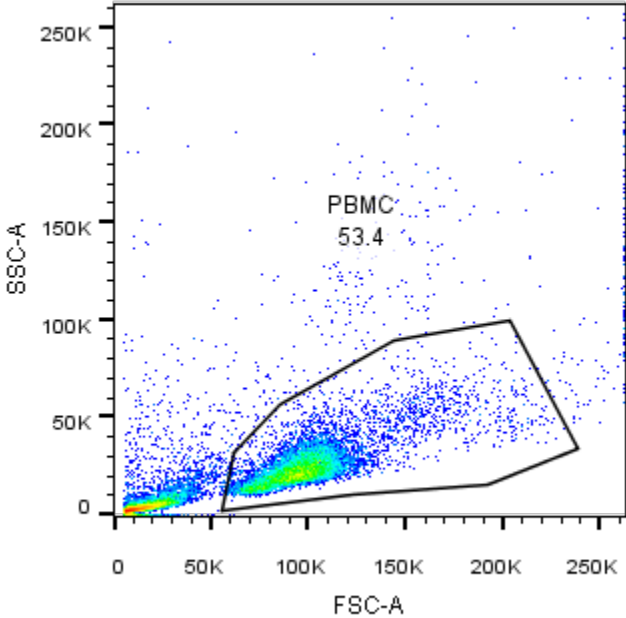
Calculate the Signal (positive Ab staining) vs the Background (negative staining):



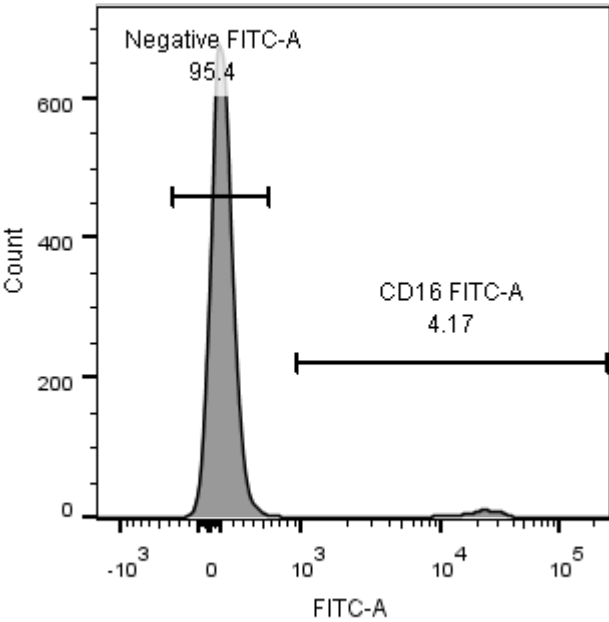
| Antibody Volume (μ l) | Signal To Background |
|----------------------------|----------------------|
| 1.25 | 1455.2 |
| 2.5 | 1168.5 |
| 5 | 1267.3 |
| 10 | 978.5 |
| 20 | 671.8 |

For CD14 PE-Cy7, the optimal antibody volume per test is 1.25 μ l due to the greater signal to background ratio. Beckman Coulter recommends 10 μ l/test.

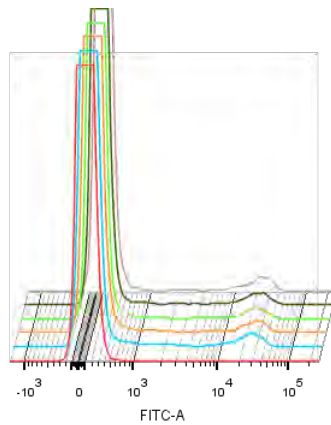
**Beckman Coulter FITC Anti-Human CD16 (clone 3G8, catalog # IM0814U)
Gate on the Mononuclear cells:**



Gate on the Positive and Negative cell populations:

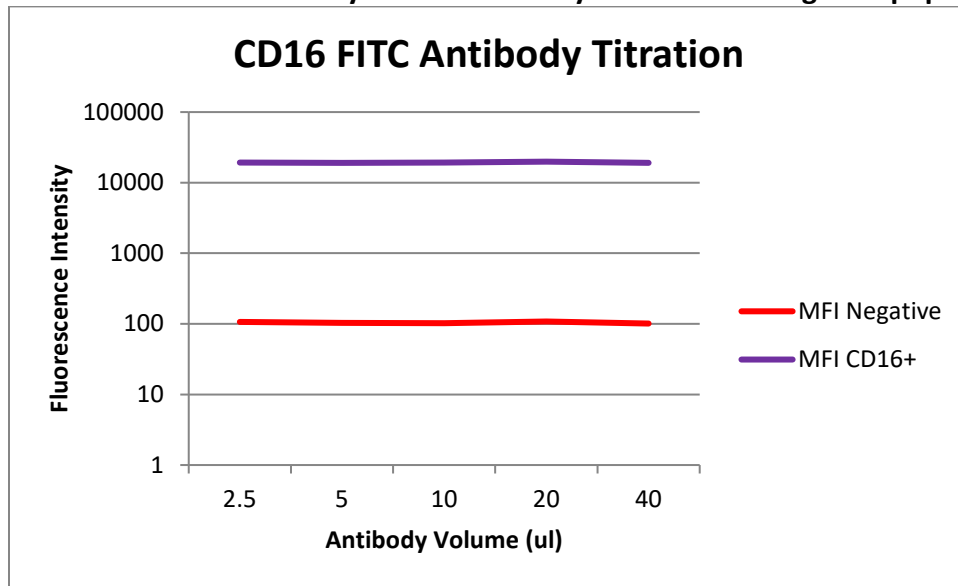


Overlay all antibody concentrations:



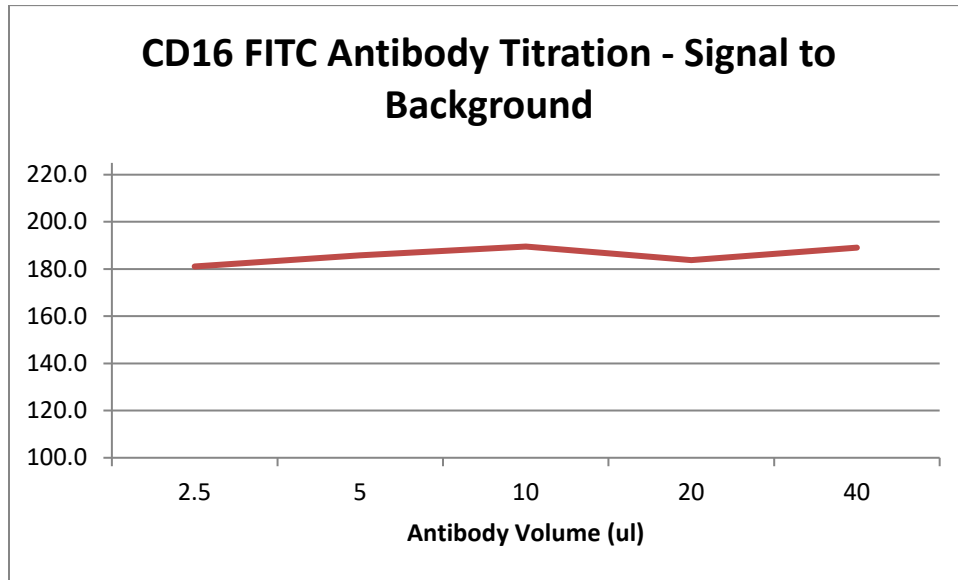
| | Sample Name | Subset Name | Count |
|---|-----------------------------------|-------------|-------|
| █ | Specimen_001_Unstained Cells.fcs | PBMC | 9690 |
| █ | Specimen_001_CD16 FITC 40 ul.fcs | PBMC | 9685 |
| █ | Specimen_001_CD16 FITC 20 ul.fcs | PBMC | 9616 |
| █ | Specimen_001_CD16 FITC 10 ul.fcs | PBMC | 9629 |
| █ | Specimen_001_CD16 FITC 5 ul.fcs | PBMC | 9655 |
| █ | Specimen_001_CD16 FITC 2.5 ul.fcs | PBMC | 9620 |

Plot the Mean Fluorescence Intensity for the Antibody Positive and Negative populations:



| CD16 FITC Antibody Titration | | |
|------------------------------|--------------|-----------|
| Antibody Volume (µl) | MFI Negative | MFI CD16+ |
| 2.5 | 106 | 19196 |
| 5 | 103 | 19136 |
| 10 | 102 | 19333 |
| 20 | 108 | 19847 |
| 40 | 101 | 19092 |

Calculate the Signal (positive Ab staining) vs the Background (negative staining):



| Antibody Volume (μ l) | Signal To Background |
|----------------------------|----------------------|
| 2.5 | 181.1 |
| 5 | 185.8 |
| 10 | 189.5 |
| 20 | 183.8 |
| 40 | 189.0 |

For CD16 FITC, the optimal antibody volume per test is 10 μ l due to the greater signal to background ratio. Beckman Coulter recommends 20 μ l/test.