# **Compensation - Using Compensation Setup**

Compensation setup is designed to work with single-stained controls. These controls can consist of single-stained cells or capture beads. We recommend to prepare a separate tube with negative beads as your negative control.

#### Start to create the compensation controls...

- after your experiment is created (means all fluorochromes are added in the parameters list and no needed fluorochromes are deleted)
- after you check your negative control sample, set up FSC and SSC

## **1. Creating Compensation Controls**

• select Experiment > Compensation Setup > Create Compensation Controls



In the "Create Compensation Controls Panel", you can add only parameters that are listed in the Parameters tab. To change to another fluorochrome for any parameter, edit cytometer settings in the Cytometer window or Inspector *before* you create compensation controls.

Create Compensation Controls		
(iii) Tubes	) Plate	
Include separate unstained control tube/we		
Fluorophore	Label	
• FITC	Generic	<u> </u>
e PE	Generic	
PerCP-Cy5-5	Generic	
PE-Cy7	Generic	
- APC	Generic	
• APC-Cy7	Generic	~
		_
Add Delete Labels	OK Cancel	

### Legend:

- Add: add a Fluorophore
- Delete: delete a Fluorophore
- Labeles..: change the Generic lable into your own name

to define antigen-specific controls while creating or modifying compensation controls

Tubes	O Plate
Include separate unstained control tube/well	
Fluorophore	Label
* FITC	Generic
e PE	Generic
PerCP-Cy5-5	Generic
PerCP-Cy5-5	CD45
# APC	Generic
# APC-Cy7	CD4
# APC-Cy7	CD8
Add Delete Labels	OK Cancel

- make sure that the "Included separate unstained control tube" is marked
- if you have labeled your fluorophores with your experiment specific antigens in the experiment layout, you'll find your label (CD or something else) **and** generic in the compensations control panel. **Delete generic ones**.
- if no label has been entered, measure under "Generic". Thus, not specifically assigned to an antigen, e.g. CD34 but to the associated fluorochrome.
- click "OK"
- a new specimen named "Compensation Control" is added and listed within your experiment
- the software automatically opens the compensation panel in the "Normal Worksheet"
- to return to your experiment ("Global Worksheet") press the button marked with 1.

📴 Browser - Experiment_008	The second secon	
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Name	Sheet1 Runstained Control FITC Stained Control PE Stained Control PerCP-Cv5-5 Stained Control PE-Cv7	
🖃 🧶 Administrator		
🚊 💼 qc		
😥 💼 Folder_001		-
😥 🔚 QC Experiment		
😥 🔚 MyExperiment	Unstained Control	
Experiment_002		-
😥 🚞 Folder_002		
🕀 🔚 6-color gating	· 동력 · P1 · 甘물	
🕀 🔚 Experiment_005		
🕀 🔚 Manual Comp_001		
Experiment_009		
Experiment_008		
Cytometer Settings	50 100 150 200 250 10 <sup>4</sup> 10 <sup>4</sup> 10 <sup>6</sup> 10 <sup>6</sup>	1
Cutometer Setting		
Cyconnecter Sectings		
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 mark the compensation tube "unstained control" (marked green) the "Unstained Control" tube displays a histogram for each fluorochrome you are going to compensate



# 1.2.1 Setting the spillover values with a <u>separate negative</u> <u>control</u>

- if you don't use a separate negative control, you have to gate also the negative cells or beads in your compensation control see page 6/7
- mix your unstained control beads tube and load it to the Sample Injection Port (SIP) press "Low" and "Run"
- click "Acquire Data" and wait a few seconds until the threshold rate is stable
- adjust gate P1 to include your population of interest
- adjust P1 to all samples, right mouse tab > "Apply to all Compensation Controls"



• check your negative bead sample in the "Unstained Control" tube, histograms should look as shown above

CS&T settings are checked weekly and define the best separation between negative and dim positive populations in a given fluorochrome chanel, therefore it is usually not necessary to change PMT voltages according to your negative beads

- look at one of your fully stained samples to see if there is any fluorochrome out of range (above 10<sup>5</sup>)
- use your single stained beads in the "unstained control" tube to see if there is any spectral overlap in any other detector



- after checking all single stained beads and setting up the PMT voltages record each single stained control in its specific tube (we recommend 20.000 events, don't use default values)
- adjust gate P2





• you can even set up the P2 Gate smaller to the Population

## 1.2.2 Setting the spillover values with <u>negative and positive beads or cells</u> in the same tube

- if you don't use a separate negative control, you have to gate also the negative cells or beads in your compensation control tubes
- mix your stained control and load it to the Sample Injection Port (SIP) Press "Low" and "Run"
- click "Acquire Data" and wait a few seconds until the threshold rate is stable
- adjust gate P1 to include your population of interest
- adjust P1 to all samples, right mouse tab > "Apply to all Compensation Controls"



- check negative bead sample in the "Unstained Control" tube, histograms should look as shown above
- look at one of your fully stained samples to see if there is any fluorochrome out of range (above 10<sup>5</sup>)
- use your single stained beads in the "unstained control" tube to see if there is any spectral overlap in any other detector
- remove the "unstained control tube/well"
  Experiment > Compensation Setup > Modify Compensation Controls



• uncheck "Include separate unstained control tube/well" and click "OK"

Modify Compensation Controls	
Tubes	O Plate
Include separate unstained control tube/well	<u>]</u>
Fluorophore	Label
• FITC	Generic
r PE	Generic
PerCP-Cy5-5	Generic
PerCP-Cy5-5	CD45
· APC	Generic
• APC-Cy7	CD4
APC-Cy7	CD8
Add Delete Labels	OK Cancel

- record data for all single stained controls in its specific tube (we recommend 20.000 events, don't use default values)
- in contrast to section 1.2.1 you have to draw a gate around the negative cells or beads in your compensation control tubes



## 1.3 Calculating Compensation

• click "Experiment" > "Compensation Setup" > "Calculate Compensation"



• the software will calculate compensation for all parameters

Single Stained Setup	
Compensation calculation has completed successfully	
Name: 200911271135	
Link & Save Apply Only Cancel	

- "Link & Save" the compensation settings will be used in your experiment and saved in the catalog ("Cytometer" > "Catalog" [allows to import compensation into other experiments])
- "Apply Only" the compensation settings will only be used in your actual experiment

• switch back to Global Worksheet by clicking on the top left icon 1.



- click on one sample tube
- make sure that the tube pointer on the far left of the sample tube is selected (green)



• check if compensation is calculated successfully

### "Cytometer" > "Compensation"

• check if "Enable Compensation" is marked

atus Parameters Thresh	old Laser Compensation Ratio	
	Enable Compensation	lear
Fluorochrome	- % Fluorochrome	Spectral Overlap
PE	APC	0.03
Alexa Fluor 488	APC	0.00
PerCP	APC	1.47
APC	PE	0.36
Alexa Fluor 488	PE	0.80
PerCP	PE	19.00
APC	Alexa Fluor 488	0.13
PE	Alexa Fluor 488	0.11
PerCP	Alexa Fluor 488	3.12
APC	PerCP	34.91
PE	PerCP	0.07
Alexa Fluor 488	PerCP	0.11

• now you are ready to collect data for your samples

# 1.4 Measure your Samples