INSTITUTO DE MEDICINA MOLECULAR JOÃO LOBO ANTUNES

PANEL DESIGN

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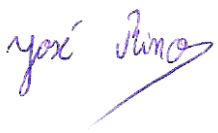

SOP.UCF.006 – PANEL DESIGN

PRINCIPLES OF PANEL DESIGN:

- Always start looking for the most critical markers;
- Match fluorochromes by brightness to the lowest density and smeared antigens;
- Minimize spillover, specially between populations/markers you want the best discrimination (xy plot);
- Use tandem dyes with consideration of their technical limitations;
- Use appropriate controls and optimize sample preparation.

MATCH FLUOROCHROMES BY BRIGHTNESS TO THE LOWEST DENSITY AND SMEARED ANTIGENS

Fluorochrome	*Laser*	Ex (nm)	Em (nm)	Filter	LP	ε (cm^{-1}M^{-1})	Quantum Yield	Brightness intensity	Brightness
PE		496,565	575	575/25	550	1960000	0.84	1646400	5
Brilliant Violet 421		405	421	450/50	-	2,500,000	0.65	1625000	5
eFluor 700 NC		660	690	670/30	-	3240000 (UV)	0.4	1296000	5
Brilliant Violet 605		405	605	610/20	600	2,400,000	0.29	696000	5
eFluor 650 NC		640	650	670/30	-	1110000	0.60	666000	5
eFluor 625 NC		610	625	630/30	600	458000	0.60	274800	5
APC		645	660	670/30	-	240000	0.68	163200	5
eFluor 605 NC		590	605	610/20	600	253000	0.60	151800	5
Texas Red			603			116000	0.9	104400	5
Cy5		649	670	670/30	-	250000	0.4	100000	5
Alexa Fluor 546		556	573	585/15	-	104000	0.96	99840	5
BD Horizon PE-CF594		488,532,561	612	610/20	600				5
Brilliant Violet 650		405	645	660/20	-	2,500,000	0.17	425000	4
Brilliant Violet 711		405	711	710/50	685	2,800,000	0.15	420000	4
Brilliant Violet 510		405	510	510/50	-	577000	0.44	253880	4
eFluor 585 NC		570	585	585/15	550	157000	0.60	94200	4
Alexa Fluor 647		650	668	670/30	-	239000	0.33	78870	4
Cy7		743	767	780/60	755	250000	0.28	70000	4
PE Cy5		496,565	670	670/30	635				4
PE Cy7		496,565	774	780/60	755				4
Brilliant Violet 570		405	570	585/42	570	2,300,000	0.08	184000	3
Brilliant Violet 785		405	785	780/60	755	2,500,000	0.04	100000	3
Alexa Fluor 568		578	603	610/20	565	91300	0.75	68475	3
Oregon Green 488		496	516	530/30	505	76000	0.9	68400	3
Alexa Fluor 488		495	519	530/30	505	71000	0.94	66740	3
Alexa Fluor 680		679	702	730/45	690	184000	0.36	66240	3
Alexa Fluor 532		532	554	530/30	505	81000	0.8	64800	3
eFluor 565 NC		550	565	575/25	550	105000	0.60	63000	3
DSRed (RFP)		558	583	585/15	-	75000	0.79	59250	3
Cy5.5		675	694	670/30	-	250000	0.23	57500	3
EYFP*		514	524	530/30	505	84000	0.63	52920	3
Venus		515	528	530/30	505	92000	0.57	52440	3
mOrange		546	562	575/25	550	71000	0.69	48990	3
7AAD		545	647	710/50	685	25000			3
APC Cy5.5		650	690	730/45	690				3
PE Cy5.5		496,565	690	710/50	685				3
PerCP Cy5.5		482	690	710/50	685				3
Alexa Fluor 660		663	690	670/30	-	132000	0.37	48840	2
Alexa Fluor 700		696	719	730/45	690	192000	0.25	48000	2
RFP Tomato		554	581	585/15	-	69000	0.69	47610	2
Alexa Fluor 594		590	617	610/20	600	73000	0.64	46720	2
FITC		493	525	530/30	505	78000	0.50	39000	2
eFluor 490 NC		470	490	510/20	495	59500	0.60	35700	2
eFluor 525 NC		505	525	530/30	505	57800	0.60	34680	2
EGFP*		489	508	530/30	505	55000	0.60	33000	2
APC/Alexa Fluor 750		650	774	780/60	755	240000	0.12	28800	2
mStrawberry		574	596	610/20	600	90000	0.29	26100	2
APC Cy7		650	774	780/60	755				2
PE Texas Red		496,565	613	610/20	600	116000			2
PerCP		482	675	670/30	635	320000			2
Hoechst Blue (33258)		352	455	450/50	-	40000	0.59	23600	1
Pacific Blue		410	455	450/50	-	29500	0.78	23010	1
Cy3		514, 547	566	575/25	550	150000	>0.15	22500	1
Indo-1 Violet (High Ca2+)		330	405	450/50	-	33000	0.56	18480	1
Marina Blue		362	459	450/50	-	18700	0.89	16643	1
mCherry		587	610	610/20	600	72000	0.22	15840	1

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DAPI			359	461	450/50	-	27000	0.58	15660	1
Alexa Fluor 555			555	565	585/15	-	150000	0.10	15000	1
Cascade Blue			398	420	450/50	-	29000	0.5	14500	1
Cascade Yellow			402	545	525/50	505	25000	0.56	14000	1
mRaspberry			597	624	610/20	600	86000	0.15	12900	1
mTangerine			568	585	585/15	-	38000	0.30	11400	1
ECFP			434	477	525/50	-	26000	0.40	10400	1
AmCyan			458	489	525/50	505	40000	0.24	9600	1
Lucifer Yellow CH			428	536	525/50	505	24200	0.21	5082	1
mBanana			540	553	530/30	505	6000	0.70	4200	1
mPlum			589	649	670/30	635	41000	0.10	4100	1
mHoneyDew			487	537	530/30	505	17000	0.12	2040	1
APC-H7			650	785	780/60	755				1
Pacific Orange			410	551	525/50	505				1
Alexa Fluor 555			555	565	575/25	550	150000			
EGFP*					510/20	495				
EYFP*					545/35	525				
Alexa Fluor 350			346	442	450/50	-	19000			??
Alexa Fluor 405			401	421	450/50	-	34000			??
Alexa Fluor 430			434	541	525/50	505	16000			??
Alexa Fluor 500			502	525	530/30	505	71000			??
Alexa Fluor 514			517	542	530/30	505	80000	0.1		??
Alexa Fluor 610			612	628	610/20	600	138000			??
Alexa Fluor 632			632	647	670/30	-	100000			??
Hoechst Red (33342)			350	650	670/30	635				??
Indo-1 Blue (Low Ca2+)			350	475	450/50	-				??
Live/Dead Aqua			367	526	525/50	505				??
Live/Dead Blue			350	450	450/50	-				??
Live/Dead Blue			750	775	780/60	755				??
Live/Dead Far Red			650	665	670/30	-				??
Live/Dead Green			495	520	530/30	505				??
Live/Dead Red			595	615	610/20	600				??
Live/Dead Violet			416	451	450/50	-				??
Live/Dead Yellow			400	575	575/25	550				??
PI (propidium Iodide)			535	620	710/50	685				??
Qdot 525			350, 405	525	515/20	-	360000			??
Qdot 545			350, 405	545	550/40	535				??
Qdot 565			350, 405	565	560/40	557	1100000			??
Qdot 585			350, 405	585	585/42	570	2200000			??
Qdot 605			350, 405	605	605/40	595	2800000			??
Qdot 655			350, 405	655	660/40	640	5700000			??
Qdot 705			350, 405	705	705/70	670	8300000			??
Qdot 800			350, 405	800	780/60	750	8000000			??

BD Biosciences Relative Fluorochrome Brightness

bdbiosciences.com/colors

This table provides general guidance with respect to the relative capability of different fluorochromes to resolve dimly stained populations; it is not a representation of absolute fluorescence. Rankings were determined by comparing the stain index (resolution) of cells stained with multiple formats on several clones run on a variety of flow cytometers. Many factors can influence the relative fluorochrome/reagent performance on a given instrument, including laser power, PMT voltage, optical filters, antibody clone, and biological sample.

Laser	Fluorochrome: Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV661 BD Horizon™ BUV737	BD Horizon™ BUV395 BD Horizon™ BUV496	BD Horizon™ BUV805
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	BD Horizon™ BV605 BD Horizon™ BV786	BD Horizon™ BV510	BD Horizon™ V450 BD Horizon™ V500
Blue (488 nm)	BD Horizon™ BB515 BD Horizon™ PE-CF594 PE-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon PE-CF594 PE-Cy5 PE-Cy7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon™ APC-R700		Alexa Fluor® 700 APC-H7 APC-Cy7

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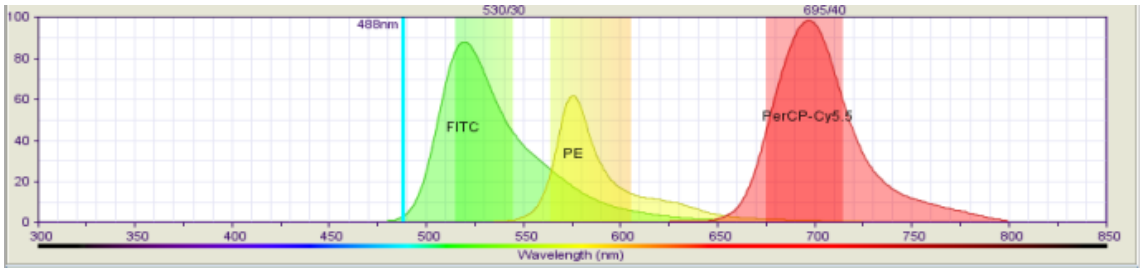
Classify the antigens you would like to measure

-**Primary**: Well characterized, easily classified as positive or negative (CD3, CD4, CD8, etc.).
-**Secondary**: Well characterized, also expressed at a higher density, often over a continuum (CD27, CD28, CD45RA/RO, IFNγ).
-**Tertiary**: Expressed at low levels only (CD25), also uncharacterized antigens.

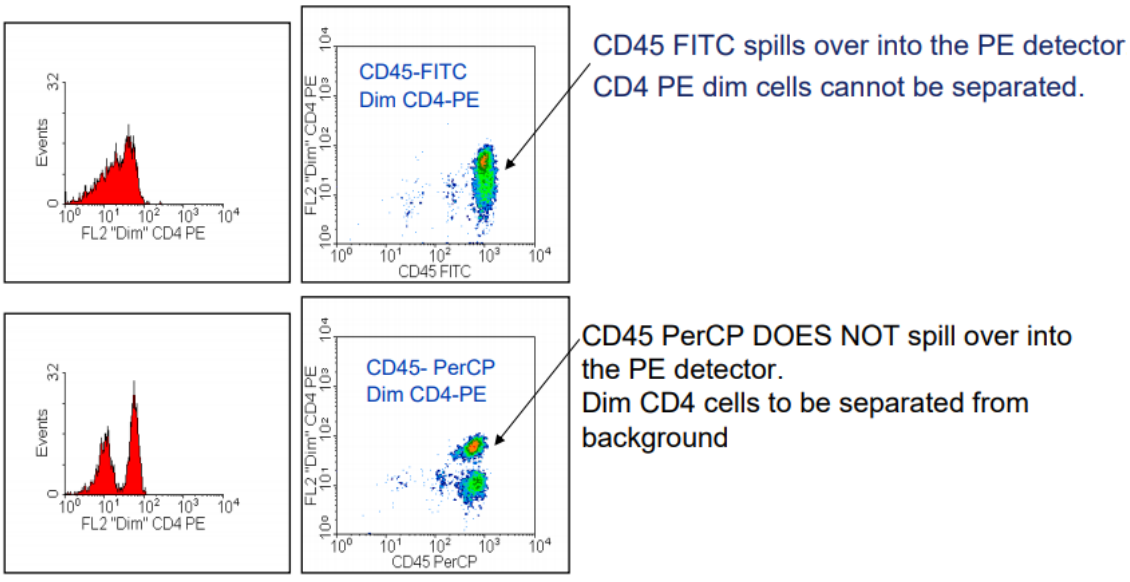
Use the brighter fluorochromes for less expressed markers; Use the dimmer fluorochromes for more highly expressed markers.

MINIMIZE SPILLOVER

Minimize the potential for spectral overlap.



CD45 **FITC** / CD4 PE vs CD45 **PerCP** / CD4 PE



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USE TANDEM DYES WITH CONSIDERATION OF THEIR TECHNICAL LIMITATIONS

• Compensation requirements for tandem dye conjugates can vary, even between two experiments with the same antibody.
– Require compensation that is lot-specific, experiment specific, and label-specific.
– Treat compensation controls the same as sample cells.
• Certain tandem dye conjugates (APC-Cy7, PE-Cy7) can degrade with exposure to light, elevated temperature, and fixation.
– Minimize exposure to these conditions.
– Use BD™ Stabilizing Fixative for final fixation.
NOTE: New tandems are more stable → APC-H7 to replace APC-Cy7

USE THE APPROPRIATE CONTROLS AND OPTIMIZE SAMPLE PREPARATION

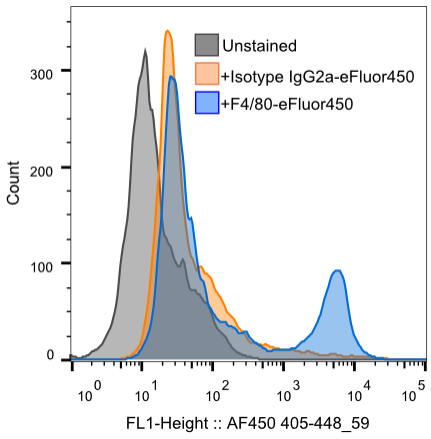
Negative control

The negative control should be a population of cells that does not express the antigen of interest. This sample should be exposed to same experimental conditions as the population in study. Use this control for setting gating regions and discerning positive from negative cells.

Isotype control

Isotype controls are used to determine the background caused by nonspecific antibody binding. An isotype control uses an antibody of the same isotype as the primary antibody, but is specific for an antigen absent from the cells under study. Isotype controls should be used to determine the background due to nonspecific antibody binding. They should not be used to distinguish positive from negative cells or set positive gating regions.

An ideal isotype control should match the primary antibody in host species, class and subclass of heavy and light chains, fluorochrome type and number of fluorochrome molecules per immunoglobulin. Also, it should be derived by the same manufacturing process and presented in the same formulation.



Fluorescence minus one (FMO) control

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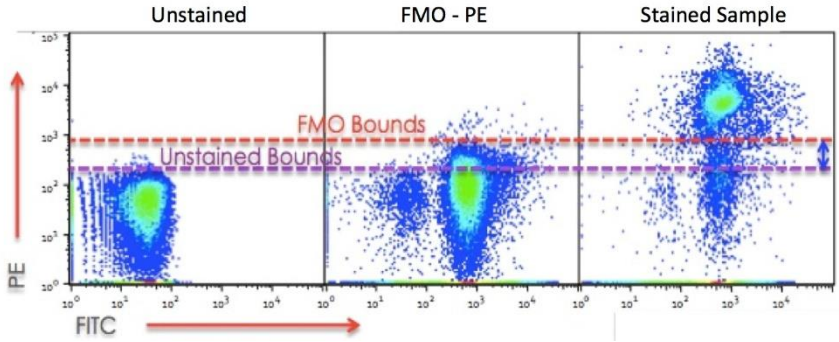
When running a multi-color flow cytometry experiment, the FMO control provides a measure of spillover in a given channel. This sample is stained with all the fluorescent conjugates except the one that is being tested, showing you the contribution of the other fluorescent conjugates in the signal of the unlabeled channel. The FMO control is fundamental in a multi-color experiment, because it allows for correct gating and select only the stained cells in the experimental sample.



Secondary antibody control

Used to determine nonspecific binding of the secondary antibody. Cells are incubated only with the secondary antibody.

Positive control

Cells known to express your target of interest. Even though they are not always available, they might be used to avoid false negatives resulting from a faulty antibody.

SAMPLE PREPARATION

- Use BSA or FBS (FACS Buffer) as a blocking agent to minimize non-specific binding.
- Use Ca/Mg++ free buffers to prevent cation-dependent cell adhesion. You can add up to 5 mM EDTA to further prevent cell adhesion. Under these conditions, BSA (0.1 – 1%) or dialyzed FBS (1 – 5%) should be used as non-dialyzed FBS would replace the Ca/Mg++.
- If studying intracellular markers, low concentrations of non-ionic detergents (up to 0.1%) are suitable to permeabilize cell membranes.
- Inclusion of 10% homologous serum or 5 mg/ml unlabelled IgG during sample staining will minimize antibody binding to Fc-receptors.
- For live cells, **internalisation of cell surface proteins can be prevented by performing all steps on ice**. Chill reagents at 4°C before use. Gentle detachments methods may be required for adherent cell lines as trypsin can induce internalisation of cell surface proteins.

- Use a **marker of dead cells** as their presence can significantly affect your analysis.
- **Titrate your antibodies to find out the optimal dilution and minimize background.** The working dilution provided in the datasheet can be used as a starting point.
- Adding **DNAse I** (25 – 50 µg/ml) and 5 mM MgCl2 will **prevent clumping** due to cell death.
- It is very important to obtain a single cell suspension to avoid clogging up the system. **Filter your samples** before the run with cell-strainer caps if possible. Use a nylon mesh only as a last resort as you may lose many cells with this system.
- Keep cells at a reasonable concentration (**1 x 10^6 – 5 x 10^6 cells/ml**). Higher cell concentrations might clog up the system and affect resolution.
- Prevent damage of the cells by avoiding bubbles, vigorous vortexing, aspirating the entire solution during buffer exchange, and excessive centrifugation.