Challenges for Flow Cytometry in Regulated Bioanalysis

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Overview

- Flow cytometry principles
- Current uses and regulatory environments
- Current regulatory guidelines
- Three Challenges

Flow Cytometry Principles

- Laser based platform used for cellular characterisation & functional analysis
- Single wavelength per laser directed onto hydrodynamically focused fluid
- Detectors measure light scatter & fluorescence emitted by cells/particles within fluid stream

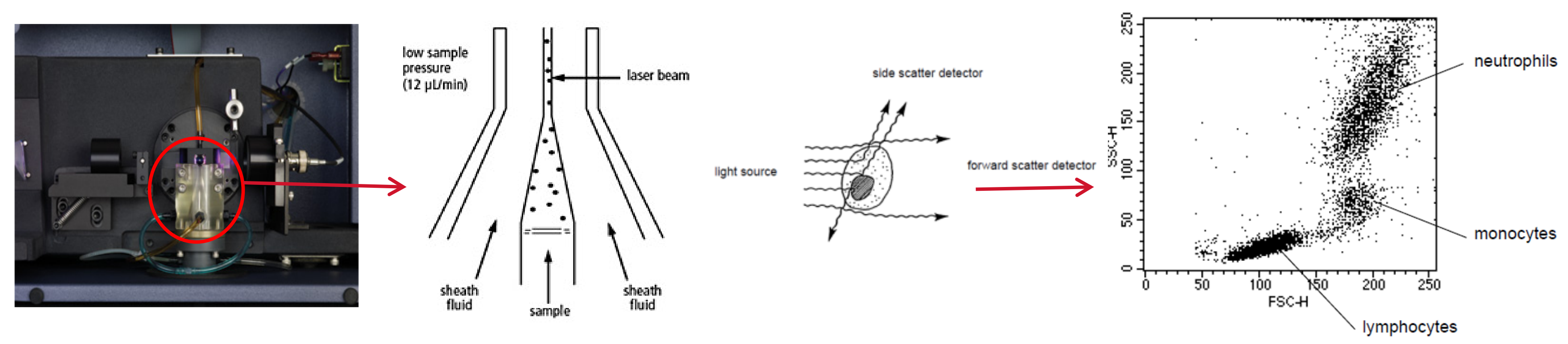
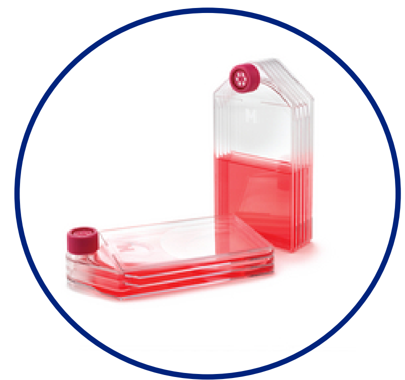


Illustration: BD Introduction to flow cytometry

Current Uses:
Drug Development Program and Regulatory environments.



Drug Discovery *In vitro*	Non-clinical Testing *Ex vivo*	Clinical Testing *Ex vivo*
Non regulated ▪ Candidate selection ▪ Mechanism of action ▪ Potency for ranking ▪ Biomarker discovery	**GLP regulated** ▪ Toxicology / Pharmacokinetics ▪ Immunogenicity **GMP regulated** ▪ Potency for batch release **Fit for Purpose/GLP** ▪ Pharmacodynamic Biomarker	**IVD FDA CLIA regulated** ▪ Drug safety & Toxicology **GLP regulated** ▪ Pharmacokinetics / Immunogenicity **GMP regulated** ▪ Potency for batch release **Fit for Purpose/GCP** ▪ Pharmacodynamic Biomarker

O'Hara, D.M *et al.*, J. Immunol. Methods (2010), doi:10.1016/j.jim.2010.09.036

Current Regulatory Guidelines:

There are numerous white papers and publications relating to the application of bioanalysis.

Anti-drug Antibody Assays (ADA):

- Mire-Sluis *et al* 2004
- Shankar *et al* 2008
- FDA (Draft) Guidance 2013
- EMA 2012

Neutralising Antibody Assays (NAb):

- Gupta *et al* 2007 and 2011
- FDA (Draft) Guidance 2013

Pharmacokinetics (PK):

- DeSilva *et al* 2003

Biomarkers:

- Lee *et al* 2006

... but none of which focus on the use of flow cytometry.

Assay Validation
Typical Parameters

- Accuracy
- Precision – Inter- and Intra-assay (+ Intra-donor)
- Sensitivity / Limit of Detection (LOD)
- Specificity / Selectivity
- Sample Stability
- Range
- Dilutional Linearity
- Drug / Matrix Interference
- Prozone effect
- Incurred Sample Reanalysis

O'Hara, D.M *et al.*, J. Immunol. Methods (2010), doi:10.1016/j.jim.2010.09.036

Challenges...

1. Reagents
2. Assay Standardisation – Instruments
3. Sample Quality and Stability

Challenge 1 - Reagents

Reagent Qualification

When reagent lots vary or degrade...

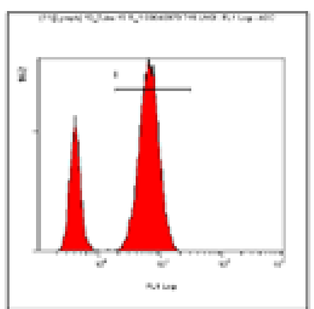
- **Reagent**: Anti-human CD4 antibody conjugated to PE-Alexafluor 750.
- **Aim:** Qualification of new lot for use in regulated sample analysis study
- **Challenge:** Qualification was not possible – apparent degradation

Challenge 1 - Reagents

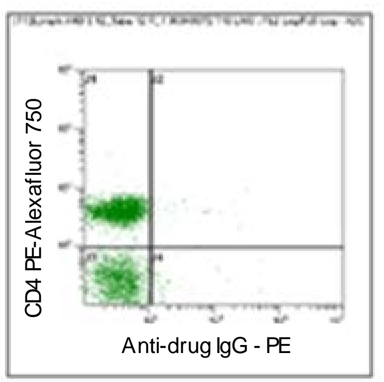
Qualification Data

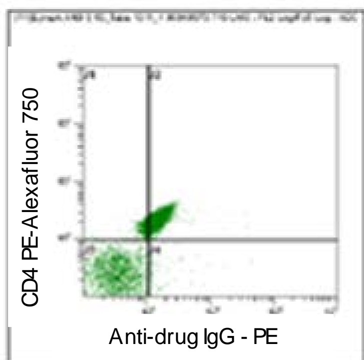
Anti CD4 PE-Alexafluor 750	Precentage CD4+ Lymphocytes	**Percentage Difference**
Lot A	39.8	**1.0%**
Lot B	40.2	

Comparable CD4+ cell detection



Similar level of fluorescence





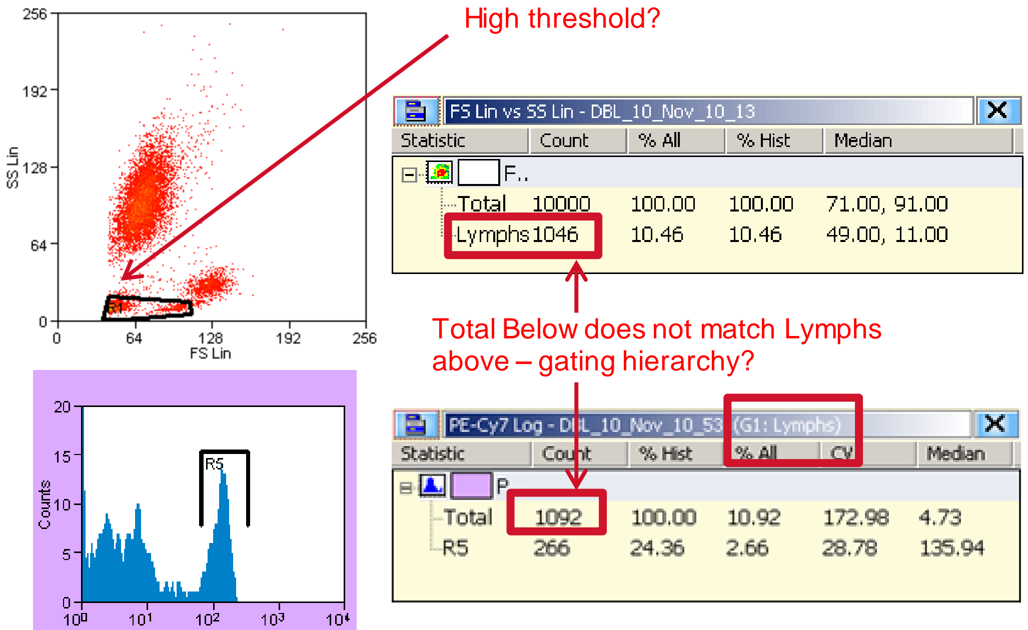
Anti CD4 PE-Alexafluor 750	Replicate	Anti-human IgG - RPE CD4 MESF
Lot A	1	700
	2	691
Lot B	1	1921
	2	1685

Footnote:
MESF - Molecules of Equivalent Soluble Fluorochrome

The reagent had degraded or was not well conjugated resulting in a bleed over of signal into the PE channel where cell bound drug was detected using an anti-human IgG PE

Challenge 1 - Reagents

Manufacturer’s Certificate of Analysis



FS Lin vs SS Lin - DBL_10_Nov_10_13

Statistic	Count	% All	% Hist	Median
F..				
Total	10000	100.00	100.00	71.00, 91.00
Lymphs	1046	10.46	10.46	49.00, 11.00

PE-Cy7 Log - DBL_10_Nov_10_53 (G1: Lymphs)

Statistic	Count	% Hist	% All	CV	Median
P					
Total	1092	100.00	10.92	172.98	4.73
R5	266	24.36	2.66	28.78	135.94

PE Log Comp vs PE-Cy7 Log Comp - DBL_10_Nov_10_53 (G1...

Statistic	Count	% Hist	% All	Median
P				
Total	795	100.00	7.95	1.85, 4.90
R8	238	29.94	2.38	4.90, 131.11
R9	0	0.00	0.00	0.00, 0.00
R10	557	70.06	5.57	1.54, 2.14
R11	0	0.00	0.00	0.00, 0.00

Under-compensated or potential degradation?

- ✘ High threshold (or discriminator) cutting off what appears to be the lymphocyte population
- ✓ Distinct positive population (CD4+ cells) from the negative population
- ✘ Note the single parameter histogram is gated on the ‘Lymphs’ however total number does not match lymph count (wrong FS vs SS plot? Or combined sets of analysis?) - Traceability
- ✘ Test sample appears to be under-compensated – similar to profile seen in qualification run

Challenge 1 - Reagents
Overview

Corrective Action:

- Supplier troubleshooting form completed
- Collaboration with the supplier to determine the cause of variation
- A new lot of reagent was manufactured which could be qualified before a larger order was placed.
- New lot was qualified and fit for use.

Overcoming Challenges:

- Systematic methods for regent qualification is critical and should correspond to the intended use of the reagent.
- Consider asking suppliers for corresponding data for reagents and compare to in-house data
- Suppliers are generally helpful and will endeavour to give you high quality reagents so asking for help may prove beneficial

Challenge 2 – Assay Standardisation

Assay Standardisation

Calibration of an assay between instruments

- **Aim:** To achieve comparable results between instruments and patient time-points and increase throughput by use of multiple instruments.
- **Challenge:** Comparability of the Median Fluorescence Intensity (MFI) between instruments was not possible even when running similar settings.
- A form of assay calibration was needed to standardise the read out from two flow cytometers.

Challenge 2 – Assay Standardisation

Inter-instrument Data

Parameter*	Replicate	Instrument 1 MFI	Instrument 2 MFI	**% Difference**
CD4^{hi}	1	26.1	8.87	**-66.0**
	2	25.4	8.79	**-65.4**
CD8^{hi}	1	24.7	8.71	**-64.7**
	2	24.3	8.55	**-64.8**

* CD4 detection using Mouse anti-human CD4:APC-eFluor 780

CD8 detection using Mouse anti-human CD8: APC

MFI – Median Fluorescence Intensity

Two instruments, same acquisition protocol and same voltage settings

↓

Difference in Median Fluorescence Intensity is observed – approximately 65% different

↓

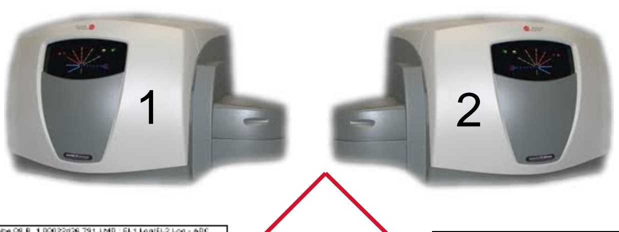
Comparable analysis of patients and time-points not possible unless performed on a single instrument.

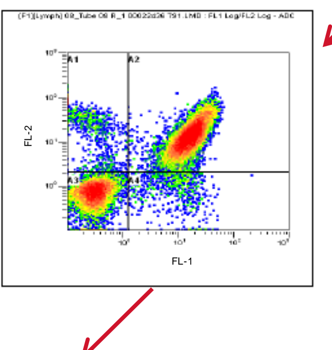
↓

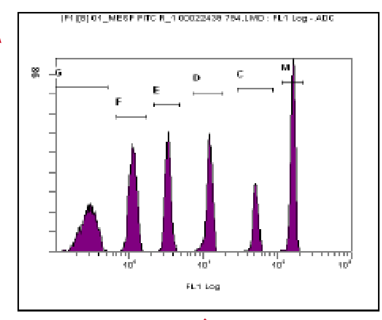
Affects the level of throughput achievable and does not account for day-to-day instrument drift.

Challenge 2

Solution Implemented







MESF – Molecules of Equivalent Soluble Fluorochrome

Parameter	Replicate	Instrument 1 MFI	Instrument 2 MFI
CD4^{hi}	1	26.1	8.87
	2	25.4	8.79
CD8^{hi}	1	24.7	8.71
	2	24.3	8.55

Calibration Plot (Normalised to 256 channels)

MESF/ABC: 100, 1000, 10000, 100000, 1000000, 10000000

Histogram Channels: 0, 60, 120, 180, 240

Parameter	Replicate	Instrument 1 MESF	Instrument 2 MESF	**% Difference**
CD4^{hi}	1	66901	64333	**-3.8**
	2	65095	63748	**-2.1**
CD8^{hi}	1	63289	63164	**-0.2**
	2	62257	61995	**-0.4**

Challenge 2 – Assay Standardisation

Corrective Action:

- Conversion of Median Fluorescence Intensity to Molecules of Equivalent Soluble Fluorochrome
- The MESF was used to calibrate the instruments to standardise the fluorescence readout
- Works by measuring the fluorescence of bead populations with known MESF
- Can account for drift of instrument, running analytical runs on different days and inter-instrument variability.

Overcoming Challenges:

- Alternative methods can be used such determination of ratios to negative populations or alignment of instruments using setup beads.

Challenge 3 – Sample Quality and Stability

Sample Logistics & Assay Set-up

Stability and Sample Processing

- **Aim:** To ensure trial sites prepare samples using the same procedures and stability of the samples is assessed for storage purposes.

- **Challenge:** Multiple trial sites in multiple countries may be used for processing samples. The challenge is in coordination of sample collection and processing activities, storage of samples and shipping to the test facility or central laboratories

Challenge 3 – Sample Logistics
Considerations

- Sample Type: For example – blood or isolated cells?
- Volume of sample required?
- Frequency of the target population – are they rare cells?
- Practical storage of samples?
- Effects of transport, storage, processing and cryopreservation on marker expression?
- Aseptic processing of samples required?

Challenge 3 – Sample Logistics

Sample Collection and Storage Method Validation

Comparison of fresh and frozen PBMC from the same donor and time-point.

The affect of cryopreservation on PBMCs was assessed to ensure the proportions of the cell types of interest were not affected by the freezing process.

The differences observed between the cell populations were <10%.

PBMC Type and Day of Analysis	Replicate	Percentage of CD4+ or CD8+ Lymphocytes	
		CD8+CD3+	CD4+CD3+
Day 1	1	75.91	98.93
Fresh PBMCs	2	75.80	98.69
Mean		75.86	98.81
Std Dev (n-1)		0.078	0.170
Precision (%CV)		0.1	0.2
Day 2	1	68.98	96.67
Frozen PBMCs	2	69.24	96.51
Mean		69.11	96.59
Std Dev (n-1)		0.184	0.113
Precision (%CV)		0.3	0.1
% Difference		**-8.9**	**-2.2**

Challenge 3 – Sample Logistics

Sample -70°C Stability Testing

Blood from 3 healthy volunteers processed to PBMCs and cryopreserved in multiple cryovials. Samples were stored at -70°C (nominal) to mimic storage at the clinical sites.

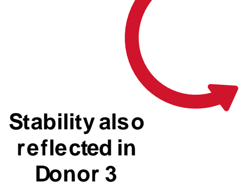
Donor 1

Stability Time point	T Cells (CD3+ of Lymphocytes)	% Difference from Baseline	Helper T Cells (CD4+ CD3+ of Lymphocytes)	% Difference from Baseline	Cytotoxic T Cells (CD8+ CD3+ of Lymphocytes)	% Difference from Baseline
Baseline	82.1	-	64.2	-	33.3	-
4 Weeks	81.7	**-0.5**	61.5	**-4.2**	35.4	**6.3**
8 Weeks	82.7	**0.7**	62.6	**-2.5**	34.8	**4.5**
12 Weeks	75.8	**-7.7**	60.7	**-5.5**	36.3	**9.0**
18 Weeks	78.8	**-4.1**	61.4	**-4.4**	35.7	**7.1**
Mean	80.2	-	62.1	-	35.1	-
Stdev	2.92	-	1.39	-	1.14	-
%CV	3.6	-	2.2	-	3.2	-

Stability also reflected in Donor 2

Donor 2

Stability Time point	T Cells (CD3+ of Lymphocytes)	% Difference from Basline	Helper T Cells (CD4+ CD3+ of Lymphocytes)	% Difference from Basline	Cytotoxic T Cells (CD8+ CD3+ of Lymphocytes)	% Difference from Basline
Baseline	60.6	-	61.0	-	32.0	-
4 Weeks	68.8	**13.4**	60.3	**-1.2**	32.4	**1.3**
8 Weeks	70.6	**16.4**	61.0	**-0.1**	32.4	**1.3**
12 Weeks	65.2	**7.6**	53.9	**-11.6**	40.5	**26.6**
18 Weeks	66.5	**9.7**	60.3	**-1.2**	32.6	**1.9**
Mean	66.3	-	59.3	-	34.0	-
Stdev	3.80	-	3.02	-	3.65	-
%CV	5.7	-	5.1	-	10.7	-



Stability also reflected in Donor 3

Donor 3

Stability Time point	T Cells (CD3+ of Lymphocytes)	% Difference from Baseline	Helper T Cells (CD4+ CD3+ of Lymphocytes)	% Difference from Basline	Cytotoxic T Cells (CD8+ CD3+ of Lymphocytes)	% Difference from Baseline
Baseline	73.1	-	57.6	-	37.6	-
4 Weeks	77.3	**5.7**	56.8	**-1.4**	38.4	**2.1**
8 Weeks	76.5	**4.6**	56.7	**-1.6**	38.7	**2.9**
12 Weeks	65.2	**-10.8**	53.9	**-6.4**	40.5	**7.7**
18 Weeks	70.8	**-3.1**	55.4	**-3.8**	39.3	**4.4**
Mean	72.6	-	56.1	-	38.9	-
Stdev	4.88	-	1.45	-	1.08	-
%CV	6.7	-	2.6	-	2.8	-

Detection of total T cells, CD4+ and CD8+ T cells is comparable for up to 18 weeks storage at -70°C (nominal). This allows up to 18 weeks storage from collection to analysis.

Challenge 3

Corrective Action:

- Aim to perform method validation (assay and sample collection) months before the start date of the trial to allow for sample stability assessments
- Assess the method of sample collection and processing: vacutainers, isolation and preservatives.
- If not using fresh samples assess the effect of cryopreservation or stabilisation of samples.

Overcoming Challenges:

- Mimic the sample processes at trial sites as much as possible for comparative analysis to allow identification and troubleshooting of poorly processed samples.
- Recommend a training program for clinical sites with sample collection guides, validated methods and possibly training videos if on site training is not possible.

Summary

Ensure a systematic qualification process for critical reagents is in place before sample analysis begins

Suppliers are generally helpful therefore consider asking for the supporting data for the certificate of analysis and compare to in-house data

Consider calibration of instruments or the assay itself to ensure consistent and reliable results. Ensure the appropriate controls are in place for comparison

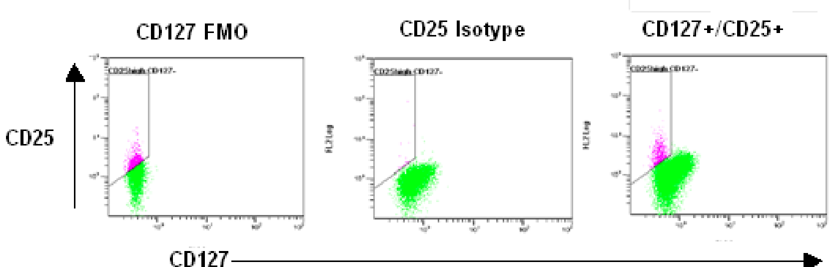
Consider standardising marker expression to a consistent parameter, do not rely solely on median fluorescence intensity

Sample coordination is often logistically challenging but these challenges can be overcome with careful planning and consideration to key aspects of the analysis.

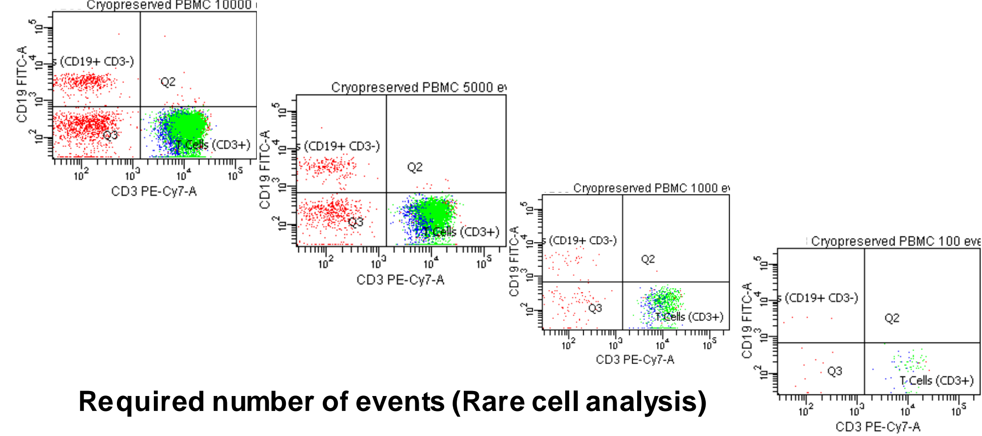
Validate the sample collection and processing methods and have the procedure officially documented for ease of trial site training and producing high quality samples.

Many other challenges....

Challenges are associated with every platform however once you get past them, flow cytometry can be a very powerful tool for a variety of applications. Overcoming your challenges will ensure high quality assays and reliable data sets....

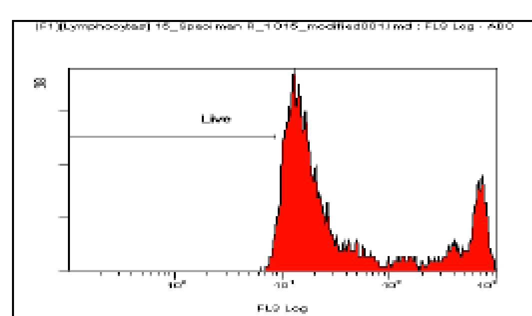


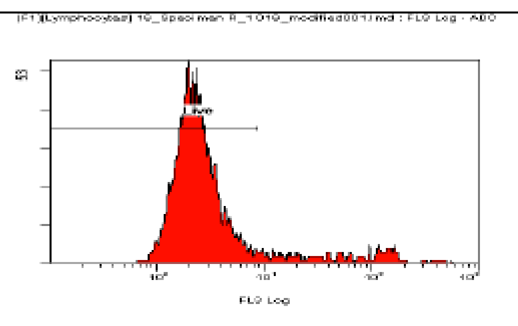
Appropriate assay controls:
Fluorescence Minus Ones and Isotype Controls
Gating controls/rules



Required number of events (Rare cell analysis)

Surrogate sample controls
Stabilised whole blood/PBMCs/Cell Lines
Assay performance control





Cell Viability Stains:
Fixable dyes, 7-AAD, PI...
Live/Dead cell discrimination