

WELLCOME SANGER INSTITUTE

STANDARD OPERATING PROCEDURE PACKET

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SANGER INSTITUTE STANDARD OPERATING PROCEDURE

SUBJECT: Peripheral blood leukocyte flow cytometry assay using whole blood

INTRODUCTION:

This SOP details the characterisation of the main leukocyte populations in peripheral blood from mice by 2 panels of 10-colour flow cytometry. The protocol is combined with cell counts obtained from a haematology analyser to derive the absolute cell numbers of various leukocyte populations.

ABBREVIATIONS:

- ARA Advanced Research Assistant
- BD Becton Dickinson
- BSA bovine serum albumin
- DCF data capture form
- EDTA Ethylenediaminetetraacetic acid
- FACS fluorescence activated cell sorting
- FSC forward scatter
- PBL peripheral blood leukocytes
- PBS phosphate buffered saline
- PIL Procedure Individual Licence
- PPE personal protective equipment
- PPL Procedure Project Licence
- RCF relative centrifugal force
- SSC side scatter

HEALTH & SAFETY:

This procedure is covered by the following risk assessment WTSI_2097

- Appropriate personal protective equipment (PPE) is to be worn at all times when handling the samples white coat and gloves
- New employees, or those returning from a significant period of absence, must be supervised until deemed competent at performing this procedure.
- Individual risk assessments for young persons and new or expectant mothers would be performed to define any exclusions for performing this assay.
- Any employees with disabilities or health issues will undertake pre-employment health questionnaires and seek advice from occupational health.
- Lone working and out of hours working is permitted for those deemed competent although out of hours should be restricted to data acquisition where possible.
- Ergonomic pipettes should be used to minimise the risk of repetitive strain injury as this process involves multiple pipetting steps.
- Users should correctly organise their workspace to minimise stretching and other ergonomic hazards.
- New users are required to be trained in the correct operation of the BD LSRII by the core flow facility staff.



- The BD LSRII flow cytometer contains class III lasers but this instrument is class I by design.
- Under no circumstances should the covers or interlocks on the instrument be overridden to access the lasers.

RESPONSIBILITIES:

All staff performing this procedure are responsible for ensuring that this Standard Operating Procedure (SOP) and accompanying risk assessment has been read and understood. All staff should be trained and competent to perform the procedure.

RESOURCES:

Equipment and reagents:

- 1. 100 μl EDTA coated blood collection tubes without capillary (Scientific Laboratory Supplies catalogue number 78042)
- 2. Centrifuge (for plates)
- 3. BD Falcon 96 well U bottom plates with lid (Sanger stores PLPL0012, BD 353077)
- 4. Compensation beads (eBioscience Ultracomp eBeads catalogue number 01-2222)
- 5. FACS buffer (PBS with 0.5% BSA)
- 1x BD Cell fix (10x Cell Fix BD Biosciences catalogue number 340181) hazardous substance contains formaldehyde and sodium azide, prepared by diluting 10x stock with ultrapure water in chemical fume hood wearing safety glasses
- 7. 1x RBC lysis buffer (10x BD PharmLyse, BD Biosciences catalogue number 555899)
 1:10 dilution with ultrapure water [remove from fridge to warm up prior to use]
- 8. Pipettes (single, multi and repeat) and tips
- 9. Formaldehyde hazardous waste container, plus funnel
- 10. Timer
- 11. 4 ml amber glass vials (Sigma catalogue number 27001-U)
- 12. 0.6 ml clear microcentrifuge tube
- 13. Antibodies (see appendix B)
- 14. Vortex
- 15. Scil Vetabc+ analyser
- 16. BD LSRII flow cytometer

Associated SOPs/Documentation:

- 1. SOP0185 Haematology Vetabc Plus QC and Sample Analysis
- 2. SOP0153 Analysis of 16 week PBL data

Staff: This procedure can be performed by one member of staff.

METHOD – Preparing working antibody solution

1. Prepare sufficient amount of antibody-mix with FACS buffer, having calculated the amount of antibodies to be used, based on titration. The tables in appendix 2 describe the staining panels.



- 2. In a 4 ml amber glass vial, pipette FACS buffer, then add antibodies as indicated for panel 1, then repeat for panel 2 in a separate vial.
- 3. Label tubes with panel, initials of person who prepared the mix and date.
- 4. Mix both tubes thoroughly by vortexing and store in fridge until required.
- 5. The antibody mix is suitable to be used for a maximum of 5 days from preparation and should be disposed of at the end of the week.

METHOD – Setting Up Compensation Controls

Note: Can be done during the 20 minute incubation

- 6. Collect compbead bottle from the fridge and mix well by vortexing
- 7. Add 6 drops of beads to a 0.6 ml microcentrifuge tube (these are a mix of binding and non-binding beads)
- 8. Mix well by vortexing and pipette 15 μl of beads into every compensation control well as indicated in the plate layout (appendix A)
- 9. Add 0.2 µl of antibody to the appropriate well (see appendix A) and mix
- 10. Incubate for 5 minutes at room temperature
- 11. Add 220 µl of FACS buffer to each well. The plate is now ready for acquisition, place in fridge until ready to analyse.

Note: Compensation controls are performed on a separate plate to the whole blood staining procedure

METHOD – Cell counting and staining whole blood

- 12. Blood is collected according to SOP0046 into 100 µl EDTA tubes.
- 13. Record the order of samples on a plate template printout.
- 14. Reverse pipette, with a filter tip, 25 μl of whole blood into two wells of a 96 well U bottom plate according to the plate layout in appendix A.
 Note: If there is insufficient blood then pipette 10 μl of whole blood per well and top up with 15 μl FACS buffer.
- 15. Start up, QC, run samples, clean and shutdown Vetabc Plus according to SOP0185. **Note**: this step should ideally be performed by a second person while the first person proceeds with the PBL assay.
- 16. Add 25 μl of 2x antibody (as prepared above) to each well using repeat pipetter and mix using multichannel pipette. To prevent contamination of the multichannel pipette, filter tips should be used at all steps.
- 17. Incubate for 20 minutes at room temperature.
- 18. In a certified chemical fume hood add 150 μl of 1x BD cell fix solution to each well and mix.
- 19. Incubate at room temperature for 5 minutes.
- 20. Centrifuge (400 rcf, 3 minutes), in a certified chemical fume hood dispose of supernatant into cell fix hazardous waste container using a funnel and a flicking action, rinse the funnel with a small amount of water.



- 21. Re-suspend pellet in 200 µl of 1x RBC lysis buffer per well. The pellet will be quite hard to re-suspend so mix the solution several times and be careful it does not splash.
- 22. Incubate for 5 minutes at room temperature, centrifuge (400 rcf, 3 minutes) and discard supernatant by flicking the waste into the white waste bucket.
- 23. Re-suspend in 200 µl of 1x RBC lysis buffer per well.
- 24. Incubate for 5 minutes at room temperature, centrifuge (400 rcf, 3 minutes) and discard supernatant by flicking the waste into the white waste bucket.
- 25. Wash with 250 μl FACS buffer per well, centrifuge (400 rcf, 3 minutes) and **discard supernatant by flicking the waste into the white waste bucket**.
- 26. Re-suspend in 250 μl FACS buffer per well. Looking from the top and from the bottom check the 96-well plate for debris and remove them with a pipette.
- 27. Add two virkon tablets to the waste liquid in the white waste bucket. Fill it to the top with tap water let it stand for a minimum of 1 hour and then flush down the sink with running water

METHOD – Data acquisition

28. At the LSRII verify the instrument is in HTS mode and the HTS is connected to the sample probe – see figures below





- 29. Log into BD FACSDiva.
- 30. Go to Experiment>New Experiment. Select the most recent experiment template.
- 31. At the end of the experiment name, add today's date in the format YYYYMMDD using the inspector window.
- 32. Check that plots are present on both 'global' and 'normal' worksheets. If they are not, open a new experiment and start again, as these cannot be retrieved at a later stage.
- 33. Open the blood plate (double click on icon in browser window) and delete sample wells as required.
- 34. Label wells with the correct mouse barcode number (in the Inspector window, click on the 'Well' tab and type the mouse ID in 'Name').
- 35. Apply application settings.
- 36. Check that the application settings have been applied, expect FSC voltage around 370, SSC voltage around 340 and FSC threshold 25,000. The application settings are linked to the blood samples but have been tested for beads.



- 37. Place the compensation plate onto HTS platform (make sure A1 is in correct position) and press the 'Run' button on the analyser.
- 38. Open the 'comp controls plate' and select the first well on the plate layout.
- 39. Acquire the first well of compensation controls and check that the beads are visible on the plots on the global worksheet (comp samples tab).
- 40. Ensure that you then select "record" to save the data.
- 41. Highlight the rest of the compensation control wells and select "run selected wells", it will record 5000 events per well as determined by the experiment layout.
- 42. In the 'normal' worksheet view, move and resize the P1 gate on the Unstained tab so it is set correctly around the beads, then right click the gate and select "apply to all compensation controls".
- 43. In each of the single stain control tabs, check that the P2 gate is set correctly around the positive bead population, most will need to be resized and/or moved to the correct population.
- 44. Calculate compensation by selecting Experiment>Compensation setup>Calculate compensation
- 45. Select "link and save" (the default file name is fine).
- 46. Place the blood plate containing samples onto HTS platform (make sure A1 is in correct position).
- 47. Open the blood samples plate and select the first panel 1 well.
- 48. Check the global worksheet is showing the panel 1 analysis, change to this if necessary.
- 49. Acquire this panel 1 sample to check the staining and that the cells are on scale.
- 50. In theory the same settings will work for both the compensation beads and the blood samples due to the way the application settings have been created, if necessary adjust FSC/SSC voltages or the FSC threshold as required.
- 51. Verify that the FSC-H vs FSC-A singlet doublet exclusion (top left plot) and CD45 vs SSC gates (top right plot) are set correctly; adjust the position of the gates as required or the voltages. You should expect >80% CD45+ events.
- 52. If this looks good record this sample, a stopping gate of 80,000 CD45+ singlet leukocyte events is set in the template.
- 53. Repeat the process for a panel 2 well, to ensure all gates are set correctly on this analysis sheet (panel 2 analysis).
- 54. Highlight the rest of the samples on the plate and click run wells
- **Note**: The plate can now be left to run but should be checked on regularly to make sure no clogs or other problems have occurred.
- 55. Export the data as an FCS file (FCS 3.0 option, default) onto a USB stick.
- 56. Run a cleaning plate on the LSRII.
- 57. When finished, <u>put the LSRII in standby mode</u>. The LSRII will continue to produce waste whilst on run mode and can overfill the waste container
 - If there are no further users booked that day, the analyser and computer can be switched off
 - Otherwise, log out of FACSDiva and PPMS and leave the LSRII on standby.
- 58. Analyse data in FlowJo X according to SOP0153



Troubleshooting

- While the plate is running, check on it periodically to make sure it is progressing as expected. If the plots start looking odd, for example, all cells start to become squashed against the bottom FSC/SSC plot axis, or very few events appear, there may be a blockage. Stop the plate as soon as you notice a problem. You can try a few things to fix this:
 - Prime the sample probe, can do this a few times (Click HTS/Prime)
 - Run a cleaning plate
 - o If in any doubt, seek advice from a member of Core facility staff
- Sample mixing and correct running can be verified by checking the dot plot showing Time on the x axis and Alexa Fluor 700 on the y axis and will normally be a flat tight line, large alterations to the flow rate will be indicated by broadening of the line or gaps appearing
- Make a note on the plate layout sheet about any issues that occur during the run. This will help when it comes to the data analysis and gives a valid reason if any results need to be QC failed
- If a blockage or other problem has caused data from some wells to be unusable, and if it can be resolved on the day, top up affected wells with 200 µl FACS buffer, mix with a pipette and run them again (select overwrite data). Normally there are still enough cells to give usable data.

	1	2		1	2	3	4	5	6	7	8	9	10	11	12
Α	US	PE- Cy7 CD11b	Α	1	5	9	13	17	21	25	29	33	37	41	45
В	FITC	BV421 NK1.1	В	1	5	9	13	17	21	25	29	33	37	41	45
С	PE	V450 Ly6G	С	2	6	10	14	18	22	26	30	34	38	42	46
D	PE- CF594 CD62	BV510 CD4	D	2	6	10	14	18	22	26	30	34	38	42	46
ш	PE- CF594 CD19	APC	E	3	7	11	15	19	23	27	31	35	39	43	47
F	PerCP -Cy5.5 abTC	Alexa 700 CD45	F	3	7	11	15	19	23	27	31	35	39	43	47
G	PerCP -Cy5.5 Ly6C	APC- Cy7 CD8a	G	4	8	12	16	20	24	28	32	36	40	44	48
Н	PE- Cy7 KLRG		H	4	8	12	16	20	24	28	32	36	40	44	48

Appendix A: plate layout

Compbeads
Samples



Appendix B: antibody panels

Panel 1

Antibody and f	fluorochrome	Channel	Dilution	Supplier	Catalogue #	Clone
CD44	FITC	FITC	2000	BD	561859	IM7
CD25	PE	PE	500	Biolegend	102008	PC61
CD62L	PE-CF594	PE-Texas Red	2000	BD	562404	MEL-14
TCRαβ	PerCP-Cy5.5	PerCP-Cy5.5	600	Biolegend	109228	H57-597
KLRG1	PE-Cy7	PE-Cy7	500	Biolegend	138416	2F1
CD161/NK1.1	BV421	Pacific Blue	600	Biolegend	108732	PK136
CD4	BV510	AmCyan	3000	Biolegend	100553	RM4-5
ΤCRγδ	APC	APC	600	Biolegend	118116	GL3
CD45	Alexa 700	Alexa 700	600	Biolegend	103128	30-F11
CD8a	APC-H7	APC-Cy7	200	BD	560182	53-6.7

Panel 2

Antibody and	fluorochrome	Channel	Dilution	Supplier	Catalogue #	Clone
Ly6B	FITC	FITC	1000	Serotec	MCA771FB	7/4
I-A/I-E	PE	PE	4000	Biolegend	107608	M5/114.15.2
CD19	PE-CF594	PE-Texas Red	2000	BD	562291	ID3
Ly6C	PerCP-Cy5.5	PerCP-Cy5.5	5000	Biolegend	128012	HK1.4
CD11b	PE-Cy7	PE-Cy7	2000	Biolegend	101216	M1/70
Ly6G	V450	Pacific Blue	600	BD	560603	1A8
lgD	BV510	AmCyan	2000	BD	563110	11-26c.2a
CD115	APC	APC	500	Biolegend	135510	AF598
CD45	Alexa 700	Alexa 700	600	Biolegend	103128	30-F11



SANGER INSTITUTE STANDARD OPERATING PROCEDURE

SUBJECT: Analysis of 16 week PBL flow cytometry data

INTRODUCTION

This document outlines the method for analysing data generated from the 16 week PBL flow cytometry screen using FlowJo software.

ABBREVIATIONS

- CD cluster of differentiation
- DCF data capture form
- FSC forward scatter
- HTS high throughput sampler
- PBL peripheral blood leukocytes
- PIL procedure individual licence
- QC quality control
- SSC side scatter
- TCR T cell receptor
- WBC white blood cell

HEALTH & SAFETY

This procedure has been classified as not containing any hazards. It does involve the use of computers and as such a DSeasy display screen equipment assessment might be required.

RESPONSIBILITIES

All staff performing this procedure are responsible for ensuring that this SOP has been read, understood and all staff should be trained and competent to perform the procedure.

RESOURCES

Equipment:

• Mac with FlowJo software installed

Staff: This procedure requires one member of staff

Associated documents:

• 16 week PBL 2panel calculation template.xltx



Arranging the Data for Analysis

- 1. Copy the raw data from the memory stick onto the Mac in an appropriate folder.
- 2. Open the FlowJo analysis template.
- 3. Drag and drop the raw data folder into the FlowJo workspace. All files in that folder that FlowJo recognises as samples will be imported and should be sorted automatically into groups for samples and compensation.

Note: If it looks like this has not happened properly, double click on the name of the groups and select the staining protocol to define the sample groups

4. Save the FlowJo file as a workspace (.wsp file) in your analysis folder with the date of the run (e.g. 16wk PBL analysis 20180918.wsp).

Checking compensation

- 5. Select the first group containing your samples, "panel 1". Right click the matrix icon mext to the first sample name and select 'Edit compensation matrix'
- 6. Enlarge the window, change preview population to 'singlets', untick 'Overlay uncompensated' box.
- 7. Click 'edit' then 'yes' in the pop up to edit the matrix
- 8. Edit the matrix numbers until you are happy that the compensation for each pair of fluorochromes looks correct. Close the window when you are satisfied (you don't need to click 'save').
 - The matrix icon in will change colour to match the new matrix so you know which has been applied
 - Do this for both panels (edit the matrix from a sample in the second group, "panel 2"). The matrix icon for the second group should then have a different colour to the first ##

Note: If an individual sample requires different compensation to the rest, right click on the coloured matrix icon in next to that sample and select 'reapply acquisition matrix'. The matrix icon will go grey in, then right click it again and select 'edit compensation matrix', follow steps 8 to 10 to change the compensation. The matrix icon then changes to a

ameren eerear ier mat eampre	te maleate ameren	e componedatori na	e been applied.
Name	Statistic	#Cells	QC
Panel 2_F9_F09_055.fcs		88856	
Singlets	97.9	86983	
Panel 2_M02338927_F06_052.fcs		88177	
Singlets	98.2	86621	
Panel 2_M02339246_H05_059.fcs		87128	
Singlets	97.5	84972	
Panel 2_M02339247_F05_051.fcs		88884	
Singlets	97.5	86697	

different colour for that sample 🗰 to indicate different compensation has been applied.



Panel 1

- FSC-A vs FSC-H (singlets)
 - CD45 vs time (HTS issues)
 - CD45 vs SSC-A (leukocytes)
 - Total T cells
 - gd T cells
 - NK cells
 - KLRG1+ mature NK cells
 - NKT cells
 - ab T cells
 - CD8+ ab T cells
 - CD4+KLRG1+ ab T cells
 - CD8+CD44+ Teff memory cells
 - CD4+ ab T cells
 - CD4+KLRG1+ ab cells
 - CD4+CD44+ Teff memory cells
 - CD4+CD25+ Tregs

Panel 2

- FSC-A vs FSC-H (singlets)
 - CD45 vs time (HTS issues)
 - CD45 vs SSC-A (leukocytes)
 - B cells
 - IgD+ mature B cells
 - NOT B cells
 - Neutrophils
 - Eosinophils (NOT neutrophils)
 - Monocytes (NOT neutrophils)
 - Ly6Chi monocytes
 - Ly6Clo monocytes

Note: On any axis, click the T icon to allow adjustment of axis scaling using the slider, making sure all events can be seen and giving better visualisation of the populations. Normally, setting the linear scale on the time plot to 120 will allow good visualisation of all the data. For all fluorescent parameters the width basis can be changed, ensuring the scale is set to BiExp

Open plots

Panel 1

9. Right click on the Panel 1 group and click the check box for synchronised, click apply changes and close. Open all plots as described in the following steps, but do not move any gates until they are all open and you have selected a representative sample to set gates on while synchronised.



10. Now the plots are synchronised the gates can be changed as described in the steps below and any changes will be applied to the whole group, minimising the number of gates that need to be changed on individual samples.

Note: once synchronised ensure the file is saved at regular intervals, changing the gates when synchronised takes a lot of memory and FlowJo can often crash at this stage

- 11. Open the first sample.
 - X axis = FSC-A; Y axis = FSC-H
 - gate = singlets
 - If this gate is not set correctly you will see double positive events in the plots which do not occur biologically e.g. cells positive for both TCRαβ and TCRγδ.



Doublets like these are what you want to avoid

- 12. Open 'singlets'.
 - X axis = Time; Y axis = Alexa700-CD45;
 - gate = Time, CD45 subset
 - Here you want to avoid any fluctuations in the event rate. This will often mean taking the middle section of the events. If the gate is hard to set or the CD161(NK1.1) vs TCRab gates are hard to set, duplicate the view and change the Y axis to Pacific Blue-CD161. As this is the lowest wavelength fluorochrome in the panel for excitation and emission this is the worst affected by an unstable flow via the HTS. From here you can determine the correct time region to use and set the gate according to this.
 - Click "T" > customize > set the max to 120. Click apply all samples.





- 13. Open 'Time, CD45 subset'.
 - X axis = Alexa700-CD45; Y axis = SSC-A;
 - gate = leukocytes
 - Ensure this gate is pushed off the top of the SSC-A axis to ensure all events are accounted for



14. Open 'leukocytes' and duplicate this new plot once more.



15. For the first plot, X axis = PerCPCy5.5-abTCR; Y axis = APC-gdTCR

- gate = Total T cells
- gate = gd T cells



16. For the second plot, X axis = PerCPCy5.5-abTCR; Y axis = PacificBlue-CD161

- gate = NK cells
- gate = NKT cells
- gate = TCRab T cells
- Between these two plots check that the sum of NKT, gd T cells and TCRab T cells is ±0.1% of Total T cells.





- X axis = PE-Cy7-KLRG1; Y axis = histogram
- gate = KLRG1+ NK cells
- This gate has been set using a FMO control and shouldn't be changed. Make a note in the QC column of the FlowJo workspace window if the whole population has shifted and is KLRG1^{hi}.



- 18. Open 'TCRab T cells'.
 - X axis = APC-Cy7-CD8a; Y axis = AmCyan-CD4
 - gate = CD8 T cells
 - gate = CD4 T cells
 - Here you should have very few CD4 CD8 double negative cells (they do not circulate) if the population is quite noticeable this might indicate the TCRab T cell gate is set too far to the left and should be adjusted





19. Open 'CD8 T cells' and duplicate this new plot once more.

20. For the first plot, X axis = PE-TexasRed-CD62L; Y axis = FITC-CD44

- gate = CD8 Teff, change the display to 'contour' using the options window if not already in this format, and check the 'show outliers' box
- This gate is quite hard to set. Use the naïve (CD62L^{hi} CD44^{lo}) and central memory (CD62L^{hi} CD44^{hi}) populations to help guide where to place the gate.
- The effector memory T cells you are after are (CD62L^{Io} CD44^{hi}). Place the gate to the left of the main population on the CD62L axis and use top of the contour for the naïve cells to determine the CD44 level (see plot below).
- Make a note in the QC column of the FlowJo workspace window if there is CD62L shedding which makes this gate hard to set. In the event of shedding the Teff gate should be moved higher on the CD44 axis to avoid the CD44^{lo} CD62L^{lo} population that is a processing artefact (this can be caused by a difficult bleed or incubation too long in RBC lysis buffer).





21. For the second plot, X axis = PE-Cy7-KLRG1; Y axis = FITC-CD44

- gate = KLRG1+ antigen experienced CD8 T cells. Change the display to 'contour' using the options window if not already in this format, and check the 'show outliers' box
- Here the gate is extended over the entire CD44 range (although the majority should be CD44^{hi}) and is set just to the right of the initial outliers from the main KLRG1 negative population.
- Make a note in the QC column of the FlowJo workspace window if the whole population has shifted and is KLRG1^{hi} that prevents the gate being placed.



- 22. Open 'CD4+' and duplicate this new plot twice 🕒
- 23. For the first plot, X axis = PE-TexasRed-CD62L; Y axis = FITC-CD44
 - gate = CD4+CD44 Teff mem
 - This gate is quite hard to set. Use the naïve CD62L^{hi} CD44^{lo} cells to help guide where to place the gate.
 - The effector memory T cells you are after are (CD62L^{lo} CD44^{hi}). Place the gate to the left of the main population on the CD62L axis and use the central point of the contour for the naïve cells to determine the CD44 level.
 - Make a note in the QC column of the FlowJo workspace window if there is CD62L shedding which makes this gate hard to set. In the event of shedding the Teff gate should be moved higher on the CD44 axis to avoid the CD44^{lo} CD62L^{lo} population that is a processing artefact (this can be caused by a difficult bleed or incubation too long in RBC lysis buffer).





24. For the second plot, X axis = PE-Cy7-KLRG1; Y axis = FITC-CD44

- gate = KLRG1+ antigen experienced CD4 T cells
- Here the gate is extended over the entire CD44 range (although the majority should be CD44^{hi}) and is set just to the right of the initial outliers from the main KLRG1 negative population.
- Make a note in the QC column of the FlowJo workspace window if the whole population has shifted and is KLRG1^{hi} that prevents the gate being placed.





25. For the third plot, X axis = PE-CD25; Y axis = AmCyan-CD4

- gate = Tregs
- If this gate is difficult to place, click on 'TCRab T cells' in the gating path to see the CD4- population and set gate to the right of this, or change the display to zebra plot using the options window (see below).

- 26. When all the gates are set correctly for a representative sample, uncheck the synchronised option for the group to allow gates to be adjusted for individual samples.
- 27. Hold Shift and click the forward button 🕨 to cycle through all the samples, checking the populations fall into the gates correctly, and tweaking any if necessary.
- 28. If staining looks abnormal add a comment to the QC column in the main FlowJo window, this will appear on the export for data analysis later
- 29. Once you have been through all samples, save the workspace file again, and close all the plots.

Panel 2

30. Open the first sample

- X axis = FSC-A; Y axis = FSC-H
- gate = singlets
- If this gate is not set correctly you will see double positive events in the plots which do not occur biologically e.g. cells positive for both CD19 and Ly6B

- 31. Open 'singlets'.
 - X axis = Time; Y axis = Alexa700-CD45;
 - gate = Time, CD45 subset
 - Here you want to avoid any fluctuations in the event rate, this will often mean taking the middle section of the events.

32. Open 'Time, CD45 subset'

- X axis = Alexa700-CD45; Y axis = SSC-A;
- gate = leukocytes
- Ensure this gate is pushed off the top of the SSC-A axis to ensure all events are accounted for

- 33. Open 'leukocytes'
 - X axis = FITC-Ly6B; Y axis = PE-TexasRed-CD19
 - gate = B cells
 - gate = not B
 - If a mouse has granulocytosis, the neutrophils are the main Ly6B^{hi} population and the size of the 'not B' gate might need to be increased to include them all

- X axis = AmCyan-IgD; Y axis = histogram
- gate = IgD+ B cells
- This gate has been set using a FMO control and shouldn't be changed.

35. Open 'not B'

- X axis = PacificBlue-Ly6G; Y axis = FITC-Ly6B
- gate = Neutrophils
- gate = Not neutrophils
- If a mouse has granulocytosis, the neutrophils can be >60% of the events on this plot and in that case the gate will need to be adjusted as best as possible

36. Open 'Not neutrophils' and duplicate this new plot once

37. For the first plot, X axis = PE-Cy7-CD11b; Y axis = SSC-A

- gate = Eosinophils
- Ensure this gate is pushed off the top of the SSC-A axis to ensure all events are accounted for
- If a sample was KLRG1 high in panel 1, it is likely it will also be shifted and appear CD11b bright for panel 2. In this case, the gate can normally be set but needs to be moved further to the right.

38. For the second plot, X axis = PE-Cy7-CD11b; Y axis = APC-CD115

- gate = Monocytes
- If a sample was KLRG1 high in panel 1, it is likely it will also be shifted and appear CD11b bright for panel 2. In this case the gate can normally be set but needs to be moved further to the right.
- This is the gate that needs to be adjusted the most in panel 2.

39. Open 'Monocytes'

- X axis = PE-I-A_I-E; Y axis = PerCP-Cy5.5-Ly6C Change the display to 'contour' using the options window if not already in this format, and check the 'show outliers' box.
- gate = Ly6C^{hi} monocytes
- gate = Ly6C^{Io} monocytes
- If there are >5% of events in the I-A/I-E high gates make a note of this in the QC column of the FlowJo workspace.

- 40. Ensure this sample looks representative and synchronise the panel 2 group to make changes to the core gates. Once set, unsynchronise.
- 41. Hold Shift and click the forward button 🕨 to cycle through all the samples, checking the populations fall into the gates correctly, and tweaking any if necessary. Once you have been through all samples, close all the plots and save the workspace.
- 42. Open the table editor **E**, this is already set up to output the data in the necessary format to excel spreadsheets, one for panel 1 and one for panel 2. Click to the right of "output" to change the destination folder to your analysis folder and add the date of the run to the file name, click "save" in this window to save the location. Then click "to file" in the table editor window to export the excel file. Select panel 2 on the left to select the panel 2 data and repeat the steps to export it.

00			FlowJo Table	es:20160519				
Table Edi	tor Edit Vis	ualize			¢	8 🖤	?	+
+	Panel 1 Panel 2	Group Panel 1 Iterate by Sample	· 🕅	Batch to Figur	e ccel ‡ od 20160509/Panel 1			
	Tables	lteration		0.	itput			
Column	Population	Statistic	Para	meter	Name			
1 💡	TUBE NAME				Mouse barcode number			
2 💡	SDATE				Assay date			
3 💡	QC							
4 Σ	singlets/Time, CD45	subs Freq. of			Total T cells			
5Σ	singlets/Time, CD45	subs Freq. of			ab T cells			

43. Save the FlowJo file and exit the programme.

Using the QC checker and calculation template

- 44. Open the panel 1 and panel 2 output spreadsheets.
- 45. Check that all barcodes match between the sheets and that they are in the same order.
- 46. Check that the following percentage columns add up to >95%: Total t cell + NK cell + B cell + monocyte + neutrophil + eosinophil

If not then there may be a gating problem somewhere.

- This could be due to low event count making the data unreliable for a panel
- If a lot of samples appear wrong the sample order between the two sheets might not be the same so go back and double check the barcodes and order
- 47. Open the "16 week PBL 2panel calculation template" file and paste in the data under the correct headings in the upper part of the "formatted and calculator" tab from the output files.

3 A 8	. C	D	E	¥	and send a	4 I	· · · · ·	. K.	la karak	M	N	0 1		9	Constant Sector	\$	Τ	U	/	X	· · · · · · ·	2	AA	A8	AC.	AD AE	AF.	AG	AH .	Al	AJ	AK	AL.	AM	AN J	AD 1	0 1	12 1	4R
1 Mouse barooAssay date	otal T cell p alp	pha beta T.CD	4+ alpha t COB+	alpha t gamm	a deltaNKT o	ell perc NK cell pe	erce-CD4+ CD3	25+ CD4+ CD44+	CD8+ CD44+ 8	LRG1+ mab CD	s+ KURG1-CD8+	KLRG1 8 cell p	ercen igD+ i	mature Monoo	cyce pelysics	hi mono Lyfk	lo mono Neut	rophil p Eosine	phil pe Panel 1 les	Ac Panel 2 leui	c Total T cel	o alpha beta T d	CD4+ alpha t C	D8+ alphat ga	mma deita hKT i	cell cour NK cell o	ount CO4+ CD	15+ CD4+ CD44+	COE+ CD44+	 x1k61+matr0 	M+ KLRG1 CD8-	 KLRG1 8 0 	ell count (gD	 mature Mor 	locyte co Lytic +	 monoclysic- 	monoc Neutr	rophil ci Eosino	ophil count
2 M02354479 09-8AV-2014	19.0	19	11	7.64	0.46	0.10	150 0	4.79	0.73	95.4	0.23	0.24	54.3	94.7	5.09	57.2	33.7	12.7	0.8 796	28 7567	140	\$7 14372	8310	\$777	349	137	182	790 298		2 1126	19	14	40745	38567	4275	2444	1441	9538	451
3 M02354480 09-8AV-2014	57.4	16.7	9.65	8.72	0.36	0.2	40 0	474	1.55	49.2	5.45	0.96	42.5	94.0	4.35	54.6	36.7	10.1	0.3 747	29 7377	130	26 12477	7213	5822	265	152	365	643 342	7	7 671	182	49	40117	43724	3267	1752	1178	7406	272
4 M02354481 09-8AV-2014	56.4	15.7	8-96	6.57	0.43	0.10	196 8	1.36 5.79	1.42	45.5	1.68	1.8	41.8	90	5.03	58.2	30.6	9.06	0.0 751	24 7462	125	19 11764	0054	4782	324	524	1475	556 MS		8 655	112	86	40105	42981	2750	2182	1156	6759	495
5 M02336295 09-8AV-2014	20.9	20.1	11.7	7.95	8.5	0.17	2.61 0	8.47 6.14	1.30	47.4	1.29	1.55	60.6	34.0	4.54	56.7	367	6.92	0.5 756	63 7639	150	30 15204	8821	6010	375	131	1596	835 542		3 660	140	95	41724	43045	2561	1905	1254	6227	421
6 M02336296 09-8AV-2014	18	17.2	9.82	6.54	0.41	0.2	194 9	175 6.49	1.69	47.7	1.49	1.65	56.3	94.9	6.28	52.8	38.3	12	0.5 752	113 7541	125	00 12913	7295	5221	312	149	1456	722 430		8 694	110	- 36	42255	40057	4712	2495	1951	9005	434
7 MI2336895 09-8AV-2014	22.4	21.5	42.3	145	- 0.7	0.01	176	12 128	1.28	52.5	1.22	4.95	877	95.2	347	42.5	32.6	10.9	0.54 764	Q4 7530	570	12 14/06	8058	6411	525	239	1336	674 681		1. 201	114	- 41	43443	41339	2766	1722	908	4293	342
8 MI2336896 09-84Y-2014	22.5	21.6	0.3	8.49	0.5	2.0	63 8	5.54	1.81	52.8	1.24	179	- 57	44	245	60	31.8	11.4	0.3 74	66 7372	987	94 16186	\$172	6637	375	142	219	744 517	- 12	6 619	154	119	41997	10494	2642	1615	456	8389	262
3 M02307576 09-8647-201	247	23.8	13	3.90	0.87	10	137 1	140 525	0.80	57.3	1.12	1.45	56.9	82.5	4.47	5/8	345	8.26	0.0	13 51/4	100	52 11/28	51.54	/304	565	187	1001	5/0 5/4		0	154	200	29432	200	2010	1337	Car.	4325	
10 M02337577 BARAV-2014	23.1	21.9	45	8.72	0.00	0.25		30 5.54	0.75	57.6	1.26	1.09	243	94	3.56	41.5	22.6	15.2	0.5 764	47 7277	1 13	51 56249	9952	0000	670	190	1967	103 529		0 758	120	- 73	20.00	37218	2590	1594	872	1990	311
11 002201218 00-0011-2011	10.1		117		875			4.99	4.12		1.15	100	20.7	80.0	3.53	00.5		8.77		11 144		90 13 147	95.70	6947	545	1991	000	1/4 440		9992	110		429.75	10100	4479	1,004	140	6176	
12 402207579 00-04-7-201	27.4	24.2	41.0	11.7	0.74	4.32	10	150 1.02	0.72	10.2	0.52	1.45	20.1	45.5	4.74	47.0	17.4	111	V.7 19	19 194		100.00	104.07	7672	558	200	104	414 411				12	110.00	14754	2967	1572	4303	4143	
1.8 LANDERSON OF MALL SEA	22.4	22.2			0.74	4.14	111	144 344	1.00	44.4	1.12	1.04		41.1	4.74	42.2	10.0	14.2	Total lau	konte	1.11		40.42	4100		100		447 444		a and			August .	helieb	aller.	Dought .	1044	10000	144
15 MILTON DAMAY, MA	243	25.2	13			4.75			1.40				10.7	87.8	3.22	16.4	94.6	7.1	localited	KULYLE	100	41 17714	9979	7745	400	-								41430	2010	1585	1011	5435	155
16 MI2337795 (05-844-2014	19.3	15.4	10.7	7.27	0.64	024 P	ercer	ntages	Pane	el 1 &	Pane	2	52.4	93.4	5.33	19.2	34.7	10.5	numbers		122	71 11722	6042	4525	278	Δh	solut	enun	nher	s Pane	118	Pan	e 2	25419	2777	1645	964	5456	225
17 MI2337796 Dh.RAV.2014	18.4	17.6	9.55	7.57	0.43	0.29	ercer	nuages	o i an	CIT G	i ane	12	52.5	85.6	4.42	65.7	35.4	3.54			100	10 13065	7273	6582	322	218	301u	enun	iber	Stan	1 T G	i an	CIZ	40542	49.70	2745	1246	7126	431
18 MI2337797 09-84Y-2014	16.8	17.5	12.4	4.29	0.44	0.22	214 9	5.42	1.67	58.8	1.42	5.12	62.3	83.6	4.27	45.5	44.9	6.2	Panel18	k Panel	2 143	41 13635	7951	5255	337	165	456	750 452	2	6 631	112	59	40648	58542	2786	1347	1250	5417	295
19 M02337798 09-84Y-251	55.1	17.3	9.01	7.18	0.35	0.17	116 8	1.67 7.13	0.57	45.6	1.42	1.27	66.2	94.3	3.39	45.5	45.1	1.0			117	21 13100	7449	5454	295	129	682	112 521	5	3 403	106	49	45490	43632	2377	1152	1096	5955	197
20 M02239105 09-864-2014	17.5	16.6	9.42	6.75	0.47	0.22 3	216 6	1.69 5.45	11	38	1.15	1.18	61	83.5	5.78	55.7	37.6	9.24	0.5 723	12 735	128	98 12245	6947	4981	348	163	1609	604 379		6 671	80	58	44795	42547	3609	2123	1431	6779	387
21 M02339109 09-8AV-2014	15.5	14.8	8.54	5.66	0.47	0.23	203 8	1.38 4.99	0.83	48.5	1.06	1.13	47.1	94.2	107	75.4	15.2	20.6	0 74	Q4 7483	115	80 10800	6399	4239	350	175	1522	536 219		5 727	62	45	21204	33187	7964	6282	1210	15361	297
22 M02339111 09-84-Y-2014	21.9	20.8	11.0	8.91	0.01	6.27	96	175 5.00	11	42.6	1.45	1.39	60.5	92	4.83	55	29	7.46	8 737	57 7263	101	63 15364	8728	6275	451	201	1458	754 443		8 605	127	87	64295	40747	3373	1256	1216	5434	342
23 MI2339582 09-8AV-2014	19.9	19.1	11.4	8.1	6.42	0.2	145 4	8.32 5.31	0.89	56	1.34	5.54	58.9	94.2	5.58	81.8	20.5	1.69	0.4 740	80 7511	543	21 14243	7909	6547	210	153	1254	737 420		2 691	106		44248	41699	4195	2554	1279	7278	326
24 M02339505 09-RAY-2014	58.7	17.8	10.1	7.44	0.64	0.2	176 1	4.54	0.51	43	1.21	1.34	62.5	93.5	2.8	62.8	28.2	9.24	0.4 762	14 7579	942	27 13676	7717	5672	238	955	1345	795 312	2	9 579	83	76	47206	64249	2879	1827	011	6996	258
25 M02339670 09-8AV-2014	16.9	16	8.56	6.99	0.40	0.19	194 8	1.54 5.54	2.21	28.7	5.27	2.13	58.7	93.9	4.01	59.2	33.6	10.7	0.8 728	67 7322	124	65 11850	8344	5166	353	140	1432	567 377	15	4 589	112	110	43725	41070	4402	2605	1401	7010	495
26 MI2339673 09-8AV-2014	17.8	16.9	9.26	7.25	0.55	02	147 9	4.45	2	52.4	1.16	1.89	573	94.1	7.91	54.2	36.3	9.04	6.7 750	40 7477	133	77 12697	6961	5438	412	148	1251	684 318	10	9 723	41	123	4355	40012	5918	3209	2151	6763	564
27 MI2339900 09-8AV-2014	26.9	25.6	13.7	11.2	0.77	6.29	239 7	7.43 5.15	0.74	58.1	1.25	1.36	53.2	94	4.35	67.2	26.7	4.69	0.4 767	43 7643	206	80 19624	10541	8561	504	301	1607	783 543		5 9967	132	116	40668	38245	3328	2236	805	6797	241
28 MI2339902 09-84Y-2014	25.4	24.2	12.8	10.8	6.73	0.20	183 0	143 558	1	54.9	9.29	1.51	54.8	94.4	2.99	60.5	31.8	10.8	0.5 765	87 756-	194	37 18531	9791	6263	558	242	1402	125 546		2 769	118	108	41401	39178	2424	5467	779	6215	414
29 M02339914 09-8AV-201	597	18.2	843		0.82	0.20	103 9	188 7.54	29	54.2	1.28	1.91	41.5	85.5	8.90	80.1	14.4	14	20 14	03 7217	147	46 13670	7209	5751	815	207	1521	112 525		60	82	100	33/50	31557	6442	5162	828	13000	210
10 M02339919 09-8AV-2019	672	16.1	8.11	7.4	0.56	03	19	10.6 10.2	3.28	91.5	1.96	1.48	459	94.4	112	73.3	19.3	-	8 741	29 734		58 11949	6014	5491	413	229	2011	635 615		0 1066	10		21995	33921	8224	6030	1585	11031	- 295
AT MELADON COMMUNICATING	15	342	141			2.5		(4)	1.19		2.54	14	24	- 10	<i>cm</i>	- 110	- 14	2	10 10			a 10.0	141/	4000		54		10 40			-		2.000	20011	2100	1/05	550	200	
33																			_					-		_	100					_		-					
14 Mount haven Assaudate	intal Total in als	tabeta T (D	A ADDA F CODA	alcha Teactor	ARING	dinum Mi call no	mb CD4+ CD3	5+ CDE+ CDE4	CORA COARA R	IBS14 matrice	N 10 8 61 COS4	0.001.0.001.0	umbe lefter	nature Motor	one in their	a monor ladd	motor Neut	until a Losine	ohi nonher	WRC court	from Vetabe																		
35 M02334479 (0-861-2010	1254	1146	663	460	28	11	54	63 32	3	90	2	1	3274	3001	343	196	116	766	52	6.0	0	W/BC	COLU	nts fr	om d	ataha	Se .												
36 N02334480 05-84Y-2016	1308	1256	725	505	27	15	138	65 34		68	20	5	4300	4456	327	179	120	760	28	7.5	2		- cou	1103 11	onna	utubu													
37 M02334481 05-84Y-2016	1384	1134	640	460	31	13	142	53 37	2	64	21	8	4462	4150	363	211	112	654	48	7.2	2		_																
38 M02336255 09-8AV-2016	1446	1392	800	550	35	12 :	146	77 50	8	60	13	9	4154	3975	321	182	118	480	39	6.9	2																		
39 M02336296 09-8AV-2016	1300	1242	710	500	30	34	140	69 46		67	31		4065	3858	453	240	178	866	42	7.2	2																		
40 M02336855 09-8AY-2016	1586	1508	871	597	50	22	125	63 63	8	65	11	6	4085	3889	260	162	85	772	36	7.0	8																		
41 M02336896 09-84Y-2016	1868	1793	1021	738	42	36	135	83 58	13	69	13	13	4731	2082	303	382	96	946	30	8.	3																		
42 M02337575 09-8AV-2016	1877	1794	908	758	51	29	104	83 52	7	60	11	11	4324	3991	340	296	117	635	30	7.	6																		
43 M02337577 09-8AV-2016	1415	1342	766	535	54	15	110	56 42	4	63	30	6	3341	3540	228	134	73	809	31	6.1	3	-																	
44 M02337578 09-8AV-2016	2585	2462	1411	997	75	23 :	287 1	118 64	2	243	36	16	5840	5649	322	196	106	903	43	20.	3																		
45 M02337579 00-841-2016	1539	1460	829	593	- 44	16	93	54 35	- 4	53		8	3312	3096	290	126	25	625	29	6.0	a	-																	
46 M02337649 09-84V-2016	1275	1230	658	541	- 20	15	112	43 37	4	100	2		2333	2153	248	138	117	452	33	4.6	2																		
#/ MU233/600 09-8AV-2016	902	656	443	380	27	2	07	30 34	5	58	6		1723	1837	382	113	27	245	11	3.8		-																	
48 M02337552 09-8AV-2016	:422	1357	762	300	30	15	140	53 44	5	71	20		3434	3387	218	143	19	415	20	5.8	0	-																	
NO AND TRACES	909	467	0.04	433		44		30		50				1000	470				~	4.1																			
51 M0/2557799 09-84V-2016	1309	1235	514	147		11	140	48 18	15	80			1140	20270	115		495	418	16	1 10																			
an another the second state	1183	1130	643	100	14		76	44 44		35	2		4333	47.74	111	107	101	114	10	50																			
# # + * Formatted and ca	culator / WE	h genetype m	6 193		100							1000					111	10		-		Det.											- 18						
	Composition and the				_		_					_	_	_	_	_	_	_		_	_						_		_			_		_		_		_	_

- 48. Auto formatting will indicate any samples where event number falls below the required minimum and QC is required. See the 'QC during analysis' section at the end of this SOP for details on these thresholds.
- 49. In the database, run the report "ABC Plus Results" filtering by assay date and sorting by mouse (ascending). Download the report to an excel file and ensure that the barcodes are in the same order as the flow data.
- 50. Copy and paste the WBC counts to the correct column in the lower part of the "formatted and calculator" tab in the "16 week PBL 2panel calculation template" file and the cell counts for each population will be automatically calculated
- 51. Paste the columns of data in the required order for upload to the database into a new file and save as a .csv file.
- 52. Upload this file to the database Note: remember to QC fail any data that was flagged in the calculation template or from your comments during FlowJo analysis.

QC during analysis

- An 'abnormal plots' or 'Equipment failure' comment can be added to with further details such as 'blockage' or 'laser issue'.
- An 'abnormal plots' comment can be combined with 'poor staining' to distinguish staining issues from analyser issues.
- The calculation template has auto formatting that highlights cells in red that are below certain thresholds for event counts to indicate that they and offspring populations should be QC failed remember to fail both the % and the cell count parameter with the comment 'insufficient events':
 - Leukocyte events < 10,000 = QC fail whole panel
 - CD4+ ab T cell count < 2000 = fail CD4+CD25+Tregs, CD4+CD44+Teffmem, CD4+KLRG1+ ab T cells
 - CD8+ ab T cell count < 1000 = fail CD8+CD44+Teffmem, CD8+KLRG1+ ab T cells
 - NK cell count < 500 = fail KLRG1+ mature NK cells
 - B cell count < 3000 = fail IgD+ mature B cells
 - Monocyte cell count < 1000 = fail Ly6Chi and Ly6Clo monocytes
- For KLRG1 shifts that meant gates could not be set, fail all KLRG1 related parameters with the comment 'KLRG1 shift, cannot set gates'

	A	В	С	D	E	F	G	Н	I	J	К
1	Mouse barco	Assay date	Total T cell percentage	alpha beta T cell percentage	CD4+ alpha beta T cell percentage	CD8+ alpha beta T cell percentage	gamma delta cell percentage	NKT cell percentage	NK cell percentage	CD4+ CD25+ alpha beta regulatory T cell percentage	CD4+ CD44+ CD62L- alpha beta effector T cell percentage
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28											
29					-	-			1		
30	-										
31	-										
32	Mouse barco	Assav date	Total T cell number	alpha beta T cell number	CD4+ alpha beta T cell number	CD8+ alpha beta T cell number	gamma delta cell number	NKT cell number	NK cell number	CD4+ CD25+ alpha beta regulatory T cell number	CD4+ CD44+ CD62I - alpha beta effector T cell number
33			=(C2/100)*(\$X34*1000)	=(D2/100)*(\$X34*1000)	=(F2/100)*(\$X34*1000)	=(F2/100)*(\$X34*1000)	=(G2/100)*(\$X34*1000)	=(H2/100)*(\$X34*1000)	=(12/100)*(\$X34*1000)	=((12/100)*(SE2/100))*(SX34*1000)	=((K2/100)*(\$F2/100))*(\$X34*1000)
34			0	0	0	0	0	(0 0	0	0
35			0	0	0	0	0	(0 0	0	0
36	1		0	0	0	0	0	(0 0	0	0
37	1		0	0	0	0	0	(0 0	0	0
38	1		0	0	0	0	0	(0 0	0	0
39			0	0	0	0	0	(0 0	0	0
40			0	0	0	0	0	(0 0	0	0
41			0	0	0	0	0	(0 0	0	0
42			0	0	0	0	0	(0 0	0	0
43	1		0	0	0	0	0		0 0	0	0
44	1		0	0	0	0	0	(0 0	0	0
45			0	0	0	0	0	(0 0	0	0
46	1		0	0	0	0	0		0 0	0	0
47	1		0	0	0	0	0		0 0	0	0
48	1		0	0	0	0	0	(0 0	0	0
49	1		0	0	0	0	0		0 0	0	0
50	1		0	0	0	0	0		0 0	0	0
51											

	L	м	N	0	Р	Q	R	S
1	CD8+ CD44+ CD62L- alpha beta effector T cell percentage	KLRG1+ mature NK cell percentage	CD4+ KLRG1+ alpha beta T cell percentage	CD8+ KLRG1+ alpha beta T cell percentage	B cell percentage	IgD+ mature B cell percentage	Monocyte percentage	Ly6Chi monocyte percentage
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32	CD8+ CD44+ CD62L- alpha beta effector T cell number	KLRG1+ mature NK cell number	CD4+ KLRG1+ alpha beta T cell number	CD8+ KLRG1+ alpha beta T cell number	B cell number	IgD+ mature B cell number	Monocyte number	Ly6C+ monocyte number
33	=((L2/100)*(\$F2/100))*(\$X34*1000)	=((M2/100)*(\$12/100))*(\$X34*1000)	=((N2/100)*(\$E2/100))*(\$X34*1000)	=((02/100)*(\$F2/100))*(\$X34*1000)	=(P2/100)*(\$X34*1000)	=((Q2/100)*(\$P2/100))*(\$X34*1000)	=(R2/100)*(\$X34*1000)	=((\$2/100)*(\$R2/100))*(\$X34*1000)
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	т	Ш	V	w	x	v	7	۵۵	AB	۵C	AD	ΔF
1	V6Clo monocyte percentage	Neutrophil percentage	Fosinophil percentage	Panel 1 leukocyte count	Panel 2 leukocyte count	Total T cell count	alpha beta T cell count	CD4+ alpha beta T cell count	CD8+ alpha beta T cell count	gamma delta cell count	NKT cell count	NK cell count
2	cybelo monocyte percentage	neutrophil percentage	cosmoprin percentage	raner i reakocyte count	runer z reakocyte count	rotar r cen count	alpha beta i celi courte	corr apia octa i con count	ebor alpha beta i celi count	guinna delta cen count	The cell count	int cen count
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32	Lv6C- monocyte number	Neutrophil number	Fosinophil number	Т	WBC count	T						
33	=((T2/100)*(\$R2/100))*(\$X34*1000)	=(112/100)*(\$X34*1000)	$=(\sqrt{2}/100)*(\$X34*1000)$	+	Weeddall	1						
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1	CD4+ CD25+ alpha beta regulatory T cell count	CD4+ CD44+ CD62L- alpha beta effector T cell count	CD8+ CD44+ CD62L- alpha beta effector T cell count	KLRG1+ mature NK cell count	CD4+ KLRG1+ alpha beta T cell count	CD8+ KLRG1+ alpha beta T cell count	B cell count	IgD+ mature B cell count	Monocyte count	Ly6C+ monocyte count
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	yec- monocyte count	Neutrophil count	Eosinophil count
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