Title: Buffy coat PBMC IFN-γ ELISA assay protocol from TRANSVAC WP4

MATERIALS

PBMC source:
PBMC from samples frozen at 1 x 10^7 PBMC/vial, to be stored in liquid nitrogen until required.

Reagent	Supplier	Catalogue number
RPMI 1640	Invitrogen	42401-042
Foetal Calf Serum, 500ml	Invitrogen	10106169
L-Glutamine, 100ml	Invitrogen	340975
96 well, U-bottomed plates	Nunc	163320
PPD-T	SSI	
PHA-M	Sigma	L8902
HBSS	Gibco	14175
Pen/Strep	Sigma	P0781
Sodium Pyruvate	Gibco	11360-039
Benzonase	Novagen	70664-3

R10: 500 ml RPMI, 50ml FCS, 5 ml Pen/strep, 5ml L-glutamine, 5 ml Sodium Pyruvate

PROCEDURE

Preparation of antigen plates

- Pre-prepare the number of antigen plates required. Below is an example template:

	1	2	3	4	5	6	7	8	9	10	11	12
A	HBSS	HBSS	HBSS	HBSS	HBSS							
B	HBSS	Neg	PPD-T	PHA	HBSS							
C	HBSS	Neg	PPD-T	PHA	HBSS							
D	HBSS	Neg	PPD-T	PHA	HBSS							
E	HBSS	HBSS	HBSS	HBSS	HBSS							
F												
G												
H												

This template is for testing 1 sample. If expecting to test more than one sample at the same time then prepare antigen plates as such. i.e. use all wells on the plate.

- Use sterile eppendorf tubes to prepare antigen dilutions at 2x working dilution as follows: (these quantities are for testing 1 sample. Scale up accordingly for multiple samples).
  a) Neg: add 300µl of assay medium (RPMI 1640 + 10% HI-FCS + 2mM L-glutamine + 1% pen/strep) to eppendorf

b) PPD-T: add 294µl of assay medium + 6µl of stock PPD-T (at 1mg/ml) for conc. of 20µg/ml

c) PHA: add 297µl of assay medium + 3µl of PHA-M (1mg/ml) for conc. of 10µg/ml

- Add 100 µl of diluted antigen or control to triplicate wells as per template.
- Add 200 µl of HBSS to surrounding wells to minimise evaporation.
- Tape lids to plates using micropore tape and store at -80°C until required.

Thawing the PBMC

- Retrieve an adequate number of vials from liquid nitrogen and put onto dry ice.
- For each PBMC used, add 10ml R10 to a 15ml tube.
- Taking one vial at a time, loosen the cap slightly and thaw it in a water bath (at 37°C) until 70% has melted. Under the hood open the cryotube, pipette the liquid content using a Pasteur pipette and transfer it to the 15 ml tube already containing R10. Add approximately 0.5ml of R10 to the cryotube and mix the content until the icy portion has completely thawed. Transfer the content to the Falcon tube.
- Repeat with the second vial of PBMC, adding it to the same tube of R10, so that all vials from the subject/time point are pooled together.
- Centrifuge at 1400rpm for 7min.
- Discard supernatant and resuspend pellet in R10 at approx $1x10^6$ PBMC per ml (10ml per vial thawed).
- Add 2µl Benzonase nuclease per ml R10 and incubate overnight at 37°C 5% CO_2 (with the lid of the tube slightly loosened).

Assay set-up

- Remove the required number of antigen plates from the -80°C freezer and defrost/warm at 37°C.
- Defrost buffy coat PBMC vials as above. Pool cells in assay medium, count and resuspended at $2x10^6$ viable PBMC per ml in assay medium.
- Add 100 µl of cell suspension (containing $2x10^5$ PBMC) to triplicate control, PPD-T and PHA-M wells of thawed, warmed antigen plates. Final antigen concentrations in 200 µl are as follows: 10 µg/ml of *Mtb* PPD (PPD-T); 5 µg/ml of PHA-M (PHA).
- Sealed plates with micropore tape and incubate at 37°C, 5% CO_2 for 72 h.

Supernatant harvest

- Take plates out of the incubator. Remove 155 µl of supernatant from each well without disturbing cells. Supernatants from triplicate wells should be pooled and stored at -80°C. Once pooled, supernatants may be split into aliquots when stored.

Title: ELISpot Protocol from TRANSVAC WP4

MATERIALS

PBMC source:
PBMC from samples frozen at 1 x 10^7 PBMC/vial, to be stored in liquid nitrogen until required.

Antigens and final concentrations:
PPD-T – 20 μg/ml
FEC – 6.25 μg/ml each peptide
SEB positive control – 10 μg/ml
Negative control – R10

Reagent	Company	Catalogue Number
RPMI	Sigma	R0883
L-Glutamine	Sigma	G7513
Pen/Strep	Sigma	P0781
Sodium Pyruvate	Gibco	11360-039
FCS (heat-inactivated)	Biosera	S1810
Benzonase	Novagen	70664-3
Casyton tubes	Sedna Sceintific	43001
Castyon counting buffer	Sedna Scientific	43003
PBS with 0.05% Tween-20	Sigma	P3563
SEB	Sigma	S4881 -1 MG
PBS (tablets)	Sigma	3813
U-bottom 96 well plates	VWR	402030716
ELISpot plates (MultiScreen-IP)	Millipore	MAIPS4510
Carbonate Buffer Capsules	Sigma	C-3041
Human IFN-γ ELISpot kit (ALP)	Mabtech	3420-2A
AP Conjugate Substrate Kit	Bio-Rad Laboratories Ltd.	170-6432

Solutions to be made up prior to starting ELISpot procedure:
Sterile
Carbonate coating Buffer (0.05M): Dissolve one capsule in 100ml dH_2O
PBS: Dissolve one tablet in 100ml dH_2O
R10: 500 ml RPMI, 50ml FCS, 5 ml Pen/strep, 5ml L-glutamine, 5 ml Sodium Pyruvate

Non-sterile
PBS with 0.05% Tween-20: Dissolve one sachet in 1 litre of dH_2O

PROCEDURE
(All work on Day 1 and 2 must be carried out in a sterile environment)

Day 1
Coating the ELISpot plate

1) Dilute anti-IFN-γ capture antibody to a final concentration of 15μg/ml in carbonate bicarbonate coating buffer (75μl in 5ml buffer is enough to coat one whole plate). Add 50μl to each ELISpot well and tap plate gently to ensure the entire surface of the well is covered.
2) Incubate the plate overnight at 4°C on a flat surface.

Thawing the PBMC

3) Retrieve an adequate number of vials from liquid nitrogen and put onto dry ice.
4) For each PBMC used, add 10ml R10 to a 15ml tube.
5) Taking one vial at a time, loosen the cap slightly and thaw it in a water bath (at 37°C) until 70% has melted. Under the hood open the cryotube, pipette the liquid content using a Pasteur pipette and transfer it to the 15 ml tube already containing R10. Add approximately 0.5ml of R10 to the cryotube and mix the content until the icy portion has completely thawed. Transfer the content to the tube.
6) Repeat with the second vial of PBMC, adding it to the same tube of R10, so that all vials from the subject/time point are pooled together.
7) Centrifuge at 1400rpm for 7min.
8) Discard supernatant and resuspend pellet in R10 at approx $1x10^6$ PBMC per ml (10ml per vial thawed).
9) Add 2μl Benzonase nuclease per ml R10 and incubate overnight at 37°C 5% CO_2 (with the lid of the tube slightly loosened).

Day 2
Washing and blocking the ELISpot plate

10) Flick out the coating solution from the ELISpot plate and add 120μl sterile PBS to each well, flick out PBS and repeat a further 4 times (5 washes in total).
11) Add 100μl R10 to each well and incubate at 37°C for 2-5h.

Making up the antigen plate

12) Antigen dilutions can be made up in a sterile 96-well U-bottomed plate (so a multi-channel pipette can be used to transfer to the ELISpot plate) or in 2ml sterile tubes.
PPD-T is at a stock concentration of 1mg/ml and needs to be diluted to 100μg/ml before being added to the ELISpot plate. So, add 20μl PPD-T to 180μl R10.
FEC is freeze-dried form containing 31.25μg of each peptide. To resuspend add 20μl DMSO and make up to 1ml with R10. Add 200μl of this to the antigen plate.

SEB (positive control) needs to be at 50µg/ml before being added to the ELISpot plate, so add 100µl stock (100µg/ml) to 100µl R10.

Counting cells

13) After 2-5 h of blocking the plate, centrifuge the buffy coat cells at 1800rpm for 5 min.
14) Resuspend pellet in 10ml R10 for counting.
15) If using a Casy Counter, add 10µl cell suspension to 10ml Casyton buffer in duplicate and count on a programme that measures cells with diameters in the range of 5.75µM – 15µM. Calculate the mean of the two counts and use this for calculating cell concentrations. A haemocytometer can also be used.
16) Centrifuge cells at 1800rpm 5 min, discard supernatant and resuspend PBMC in R10 at a concentration of 3.75×10^6 /ml. This will give 300,000 PBMC per 80µl. Take 600µl of this cell suspension and add to 600µl R10 to make a 1 in 2 dilution of cells. Use this extra cell suspension for PPD stimulation and neg control
17) Flick out blocking solution from the ELISpot plate, do not wash and add 80µl cell suspension per well. If there are enough cells, do 6 replicates for each antigen and positive and negative controls (see plate layout below).
18) Add 20µl of antigen (from antigen plates made up earlier) to corresponding wells. Add 20µl R10 to each of the negative control wells.

	1	2	3	4	5	6	7
A	PPD-T	PPD-T	PPD-T	PPD-T	PPD-T	PPD-T	
B	PPD-T	PPD-T	PPD-T	PPD-T	PPD-T	PPD-T	1 in 2 cell dilution
C	FEC	FEC	FEC	FEC	FEC	FEC	
D	SEB(pos)	SEB(pos)	SEB(pos)	SEB(pos)	SEB(pos)	SEB(pos)	
E	neg	neg	neg	neg	neg	neg	
F	neg	neg	neg	neg	neg	neg	1 in 2 cell dilution

19) Incubate the plate for 18-20h at 37°C, 5% CO_2

<u>**Day 3**</u>

20) Flick out PBMC from ELISpot plate and wash 5 times with PBS, containing 0.05% Tween 20.
21) Dilute biotin anti-IFN-γ Ab 1 in1000 in PBS and add 50µl of dilution to each well. Incubate for 2 h at room temperature.
22) Flick out plate and wash 5 times with PBS, containing 0.05% Tween 20.
23) Dilute streptavidin-ALP 1 in 1000 in PBS and add 50µl of dilution to each well. Incubate for 1 h at room temperature.
24) Flick out plate and wash 5 times with PBS, containing 0.05% Tween 20.

25) Add 50 µl of Developing Buffer to each well and incubate for approximately 10 min at room temperature until distinct spots develop.

1 ml Working Developing Buffer Solution (1 ml)

940µl Water	(47µl / well)
40 µl Developing buffer stock	(2 µl / well)
10 µl Solution A	(0.5 µl / well)
10 µl Solution B	(0.5 µl / well)

26) Terminate the substrate reaction by washing the plate in tap water 3-4 times.
27) Leave to dry overnight.
28) Read on an automated ELISpot reader (AID version 5.0) with the following settings:

Camera settings	
Brightness	0
Sharpness	0
White balance(R)	137
White balance (B)	130
Hue	120
Saturation	155%
Pan	0
Gamma	129
Shutter (exposure)	2395
Gain	2
Iris	0
Focus	0
Zoom	0
Tilt	0
Features control	
Autoexposure	128

TB Settings				
Intensity	min	15	max	not ticked
Size	min	10	max	not ticked
Gradient	min	2	max	not ticked
Emphasis		small		

Criteria for passing the ELISpot plate

i) Positive control: SEB is used as positive control, 200 spots must be present in at least one of the SEB wells. If the positive control fails and any of the other test wells contain 200 spots, then this can be considered as a positive control for the assay.

ii) Negative control: Negative control wells containing only complete medium and PBMC must have under 20 spots per well.

DATA ANALYSIS

For each test antigen, results are reported in SFC/million PBMC. This is calculated by subtracting the mean number of spots in the negative control wells from the mean number of spots in the antigen wells. This number is then divided by 0.3 to give a result in SFC/million PBMC. Wells are considered positive if the mean count was at least twice that in the negative control and at least 5 spots more than the negative control wells.

Title: Human intracellular cytokine staining (ICS) protocol from TRANSVAC WP4

MATERIALS

PBMC source:
PBMC from samples frozen at 1 x 10^7 PBMC/vial, to be stored in liquid nitrogen until required.

Reagent	Supplier	Catalogue number	mAb clone
RPMI	Sigma	R0883	-
L-Glutamine	Sigma	G7513	-
Pen/Strep	Sigma	P0781	-
Sodium Pyruvate	Gibco	11360-039	-
FCS (heat-inactivated)	Biosera	S1810	-
Benzonase	Novagen	70664-3	-
SEB	Sigma	S4881	-
PPD	SSI	2391	-
FEC	NIBSC		-
Brefeldin A	Sigma Aldrich	B7651	-
Vivid LIVE/DEAD	Molecular Probes, Invitrogen	L34955	-
CD14 eFluor® 450	Ebioscience	48-0149-42	61D3
CD19 eFluor® 450	Ebioscience	48-0199-42	HIB19
CD4 APC-Cy7	Biolegend	317418	OKT4
CD3 PerCP	Biolegend	300428	UCHT1
CD8 FITC	Biolegend	300906	HIT8a
IFNγ PE	Caltag Medsystems	MHCIFG04	B27
Compbeads	BD	552843	-
PBS	Sigma	3814	-
Sodium azide	Sigma Aldrich	S2002	-
BSA	Sigma Aldrich	A7906	-
Fix/perm and wash buffer	BD	554723	-
Paraformaldehyde	Alfa Aesar	43368	
5mL FACS tubes (Sterile)	Falcon	352054	-
5mL FACS tubes with filter cap	Falcon	352235	-

R10: 500ml RPMI, 5ml Pen/Strep, 5ml L-glutamine, 5ml Sodium Pyruvate
FACS buffer: 0.1% BSA, 0.01% Sodium azide in PBS (made by dissolving 1 PBS tablet in 100ml dH_2O). Filter and store at 4°C.
Perm/wash buffer: Dilute 10x stock 1:10 in dH_2O to give a 1x working concentration.

PROCEDURE

All work on Day 0 and 1 must be carried out in a sterile environment

I. Stimulation of PBMC

Day 0: Preparation of cryopreserved PBMC for stimulation

1. Retrieve an adequate number of vials from liquid nitrogen and put onto dry ice.
2. For each PBMC used, add 10ml R10 to a 15ml tube.
3. Taking one vial at a time, loosen the cap slightly and thaw it in a water bath (at 37°C) until 70% has melted. Under the hood open the cryotube, pipette the liquid content using a Pasteur pipette and transfer it to the 15 ml tube already containing R10. Add approximately 0.5ml of R10 to the cryotube and mix the content until the icy portion has completely thawed. Transfer the content to the tube.
4. Repeat with the second vial of PBMC, adding it to the same tube of R10, so that all vials from the subject/time point are pooled together.
5. Centrifuge at 1400rpm for 7min.
6. Discard supernatant and resuspend to around $2x10^6$ to $3x10^6$ PBMC/ml R10 in 15 ml tubes (one tube per subject per time point i.e. one tube per vial unless pooling duplicate vials).
7. Add 10U/ml benzonase (2µl/ml).
8. Rest overnight with loose caps at 37°C in 5% CO_2.

Day 1: Stimulation

9. Pre-warm R10 in 37°C waterbath.
10. Wash PBMC in warmed R10 and count cells, spin at 1500 RPM, 5 min.
11. Resuspend PBMC to $1x10^6$ PBMC/ml in supplemented R10.
12. Aliquot 1ml ($1x10^6$) PBMC into labelled 5ml FACS tubes containing antigen e.g.
    1x PPD (20µg/ml working concentration from a stock of 1mg/ml)
    1x unstimulated
    1x SEB (5µg/ml working concentration from a stock of 1mg/ml)
    FEC (25µg /ml working concentration from stock of 1mg/ml stock)
13. Incubate with loose caps at 37°C in 5% CO_2 incubator for 2 h.
14. Add 3µg/ml Brefeldin A (from a stock of 5mg/ml DMSO, dilute 1:10 in PBS and add 6µl/tube) and incubate with loose caps at 37°C in 5% CO_2 for 16 h in incubator (overnight).

II. Day 2: ICS staining of stimulated PBMC

All staining performed in the same 5ml FACS tubes used for stimulation.

Prepare Surface and ICS mixes as in Table 1 and 2 below.

1. Wash x1 with 2-3 ml FACS buffer at 1500 RPM 5 min.
2. Check for pellet before pouring off supernatant and blotting remainder on tissue. Vortex pellet gently to resuspend cells.

3. Prepare 1:40 dilution of vivid with dH_2O. Add 5μl of diluted vivid to each test. Vortex gently. Incubate at 4°C for 10 min in dark.
4. Remove cells from fridge. Do not wash.
5. Add 9μl of the pre-prepared Surface master mix (Table 1).
6. Incubate at 4°C for 30 min in dark.
7. Meanwhile, prepare compensation controls (comp beads) during surface antibody incubation of test samples:
    - Add 2 drops of negative and positive beads (each), stain with 2μl of antibody in each case.
    - Incubate 20 min in the dark at 4°C.
    - Wash 1X with 2ml FACS buffer, 1500 RPM, 5 min.
    - Deacant supernatant and re-suspend in 200μl FACS buffer.
    - Store at 4°C in the dark until acquisition.
8. Wash cells x1 with 2-3ml FACS buffer at 1500 RPM, 5 min. Check for pellet before pouring off supernatant and blotting remainder on tissue. Vortex pellet gently to resuspend cells.
9. Add 250μl (neat) fix/perm, vortex gently and incubate at 4°C for 20 min in the dark.
10. Wash x1 with 2-3ml perm/wash buffer at 1800 RPM, 5 min. Check for pellet before pouring off supernatant and blotting remainder on tissue. Vortex pellet gently to resuspend cells.
11. Add 15μl of ICS master mix to each sample (Table 2). Incubate at room temperature for 30 min in the dark.
12. Wash x1 with 2-3ml of perm/wash buffer at 1800 RPM, 5 min. Check for pellet before pouring off supernatant and blotting remainder on tissue. Vortex pellet gently to resuspend cells.
13. Add 250μl of FACS buffer/1% paraformaldehyde (to fix cells). Vortex and wrap in aluminium foil.
14. Store at 4°C in the dark until acquired – within 24 h.
15. Immediately prior to acquisition transfer cells into filter-cap FACS tubes (to remove cell clumps).
16. Before acquiring samples on the LSR-II, run CS&T bead for performance tracking.
17. Run and record compensation tubes and unstained cells.
18. DO NOT compensate while acquiring, do this when you analyse your data using flowjo (Treestar, USA).
19. Run all the cells in each sample. Avoid running dry.

Table 1: Surface master mix, vortex and microfuge briefly:

CD14 eFluor® 450	2μl per test
CD19 eFluor® 450	2μl per test
CD4-APC-Cy7	5μl per test
Total volume per test	**9 μl**

Table 2: ICS master mix, vortex and microfuge briefly:

CD3-PerCP	5μl per test
CD8 FITC	5μl per test
IFNγ-PE	5μl per test
Total volume per test	**15μl**

Note: Antibody titrations should be performed on every new vial.

Title: IFN-γ ELISA protocol from TRANSVAC WP4

MATERIALS

Reagent	Supplier	Catalogue number
Purified mouse anti-human IFNγ ELISA capture antibody, 1 mg/ml	BD	551221
Biotinylated anti-human IFNγ ELISA detection antibody, 0.5mg/ml	BD	554550
Recombinant human IFNγ ELISA standard, 25μg	BD	554616
Avidin Peroxidase, 5mg	Sigma	A3151
OPD Fast, 50 sets	Sigma	P9187
Foetal Calf Serum, 500ml	Invitrogen	10106169
AB serum, 100ml	Sigma	H4522
$NaHCO_3$, 500g	Sigma	S5761
Tween 20, 500ml	Sigma	P1379
PBS tabs, 100 per box	SLS	BR00149
RPMI, 10x500ml	Invitrogen	42401-042
Immulon 4 HBX ELISA plates	ThermoLab	735-0465
2M H_2SO_4		
Distilled water		

PROCEDURE

Day 1 – Coating of plates

- Label ThermoLab Immulon 4 HBX plates with assay number and assay cytokine.
- Prepare Carbonate coating buffer [0.1M $NaHCO_3$, pH 8.2]. (Add 2.1g $NaHCO_3$ to 250ml dH_2O and filter)
- Dilute purified anti-human IFN-γ mAb at a final concentration of 2μg/ml (1/500 of current stock) in coating buffer. (i.e. use 10μl in 5ml per plate)
- Add 50μl/well (except well A1 – the blank), and tap to ensure the surface is covered. Cover with plate sealer.
- Incubate overnight at 4°C.

Day 2

- Defrost heat-inactivated foetal calf serum (HI-FCS) and heat-inactivated human AB serum (HI-AB).
- Prepare washing solution [PBS + 0.05% Tween-20 (P/T)].
- Wash plates x4 with the plate washer set to fill 350μl, and soak for 10 sec (also possible to perform by hand with a multichannel if only using a few plates).

- Prepare Blocking solution [PBS (from tablets) + 10% HI-FCS (PBS/FCS)].
- Add 150μl blocking solution per well (except A1 – the blank).
- Incubate for 2 h at room temperature (RT).
- Remove supernatant storage plates, IFN-γ standard and positive control from -80°C freezer at the beginning of the incubation.
- Prepare standard diluent [RPMI + 5% HI-AB, filtered through 0.2μm acrodisc syringe filter].
- Prepare the standard curve by serial doubling dilution from 4000 to 31 pg/ml, using the standard diluent. (1/250 for top standard i.e use 20μl standard at 1μg/ml in 5ml diluent).
- Dilute sample supernatants in standard diluent to give 100μl each of "neat", 1/3, 1/9, and 1/27 dilutions
- Wash plates x2 with the plate washer set to fill 350μl, and soak for 10 sec (or by hand)
- Add 50μl per well of sample, standards and controls all in duplicate, as shown on the template. Each plate should be done in sequence with the standards and samples added before moving onto the next plate.
- Incubate overnight at 4°C.

DAY 3

- Dilute the detection antibody – biotin mouse anti-human IFN-γ mAb to a final concentration of 1μg/ml in blocking solution (PBS/10% FCS) (a 1/500 dilution of current stock). (i.e. use 20μl in 10ml per plate)
- Wash plates x4 with the plate washer set to fill 350μl (or by hand), and soak for 10 sec
- Add 100μl per well (except A1 – the blank) of detection antibody.
- Incubate for 45 min at RT.
- Defrost Avidin Peroxidase (-70°C freezer)
- Dilute Avidin Peroxidase to produce a final concentration of 2.5μg/ml in PBS/FCS (a 1/400 dilution of 1mg/ml stock).
- Wash plates x 4 with the plate washer set to fill 350μl (or by hand), and soak for 10 sec
- Add 100μl per well (except A1 – the blank) of Avidin peroxidase.
- Incubate for 30 min at RT.
- Prepare OPD Fast solution by dissolving both tablets in dH_2O (for 2 plates dissolve both tablets in 20ml dH_2O) – (dissolve buffer tablet first which takes a while with shaking. Dissolve the OPD tablet just prior to use)
- Wash x5 (the first and last washes being by hand by filling wells with a squidgy bottle, the 2nd, 3rd and 4th washes with the plate washer set to fill 350μl (or with a multichannel), and soak for 10 sec).
- Add 100μl OPD Fast solution to all wells (including the blank) and develop in the dark. (reaction usually takes 10-15 min)
- Stop with 50μl/well 2M H_2SO_4 (when the 4000pg/ml well reaches a dark orange colour, and when the 31pg/ml has begun to show some change in colour).
- Read plate at 492nm.

Example template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	4000	2000	1000	500	250	125	62.5	31.25		NC	PC
B		4000	2000	1000	500	250	125	62.5	31.25		NC	PC
C	Neg	PPD-T (N)	PPD-T (1/3)	PPD-T (1/9)	PPD-T (1/27)	PHA	Neg	PPD-T (N)	PPD-T (1/3)	PPD-T (1/9)	PPD-T (1/27)	PHA
D	Neg	PPD-T (N)	PPD-T (1/3)	PPD-T (1/9)	PPD-T (1/27)	PHA	Neg	PPD-T (N)	PPD-T (1/3)	PPD-T (1/9)	PPD-T (1/27)	PHA
E	Neg	PPD-T (N)	PPD-T (1/3)	PPD-T (1/9)	PPD-T (1/27)	PHA	Neg	PPD-T (N)	PPD-T (1/3)	PPD-T (1/9)	PPD-T (1/27)	PHA
F	Neg	PPD-T (N)	PPD-T (1/3)	PPD-T (1/9)	PPD-T (1/27)	PHA	Neg	PPD-T (N)	PPD-T (1/3)	PPD-T (1/9)	PPD-T (1/27)	PHA
G												
H												

Blank	A1
Standards	A2-9 B2-9
Controls	A11-12 B11-12
Sample 1	C1-6 D1-6
Sample 2	E1-6 F1-6
Sample 3	C7-12 D7-12
Sample 4	E7-12 F7-12