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Genetic and biochemical characterization of the cytochrome P450, CYP6P9, associated with pyrethroid resistance in the African malaria vector Anopheles funestus

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APPENDICES

Appendix A1: **Protein standard curve (Bradford, 1976)**

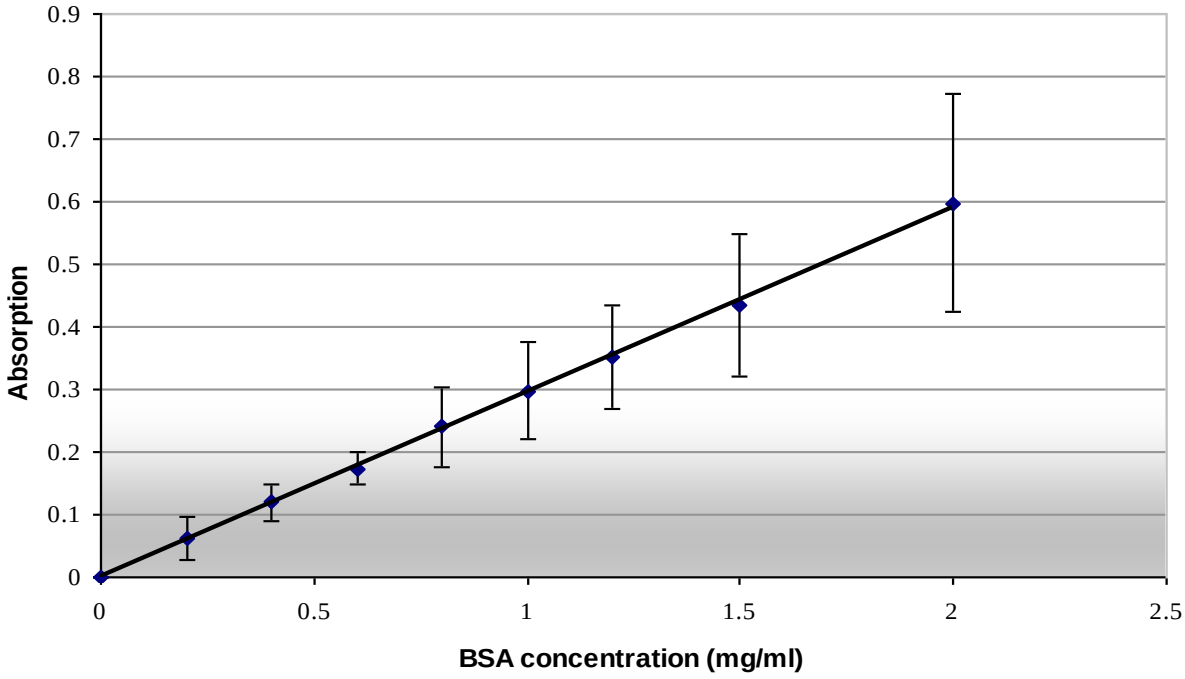

Figure A1: Protein standard curve. Bradford's reagent was use to determine the concentration of P450 Protein at A595nm. The curve had an R^2 value of 0.9994 and the equation used was y= 0.2956x – 4E-05

Appendix A2 - **Reproducibility of qRT-PCR tested for CYP6P9 on the Light Cycler® 480**

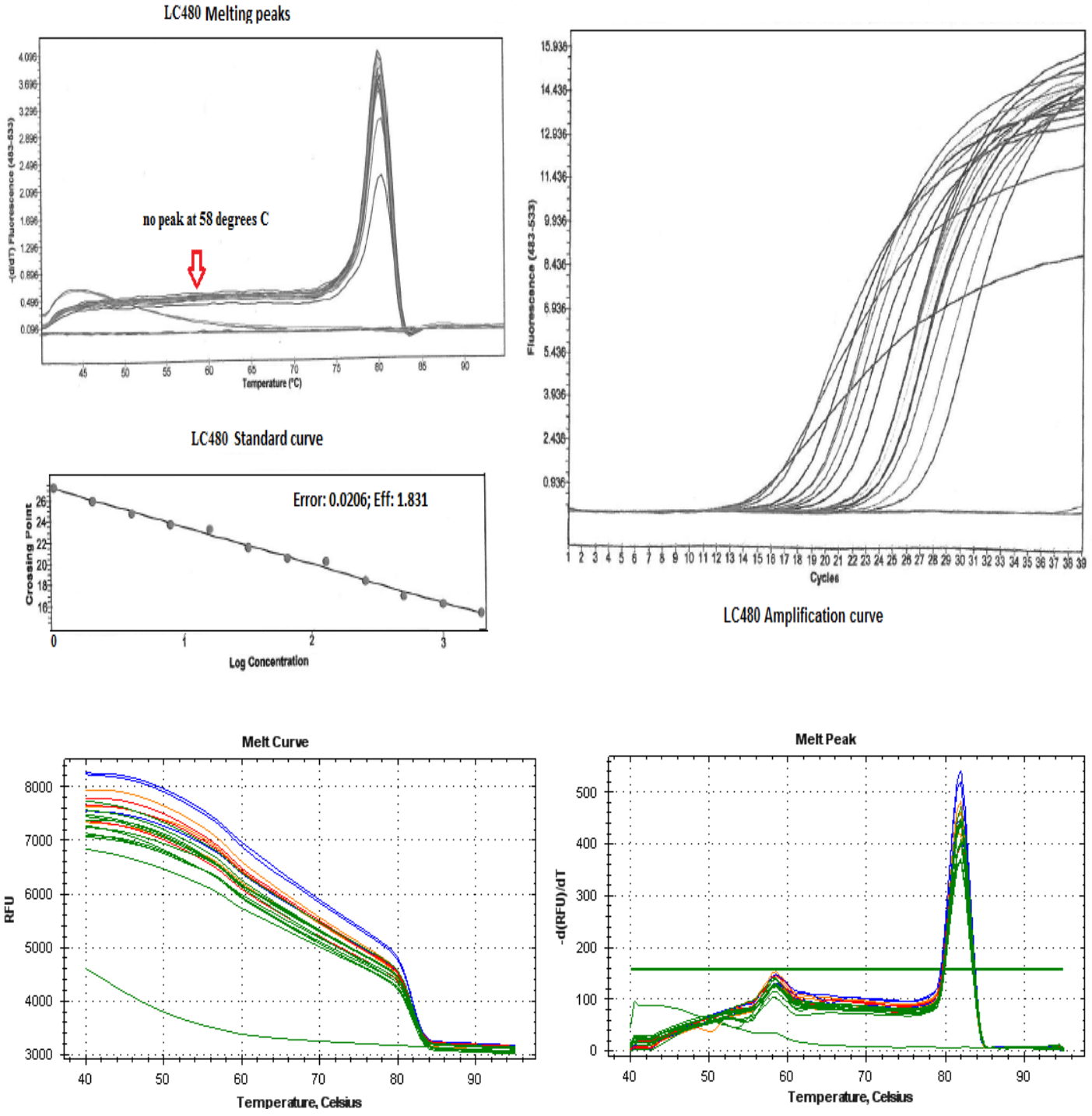

Figure A2-1: **CYP6P9 amplification profile, melting peak and standard curve** - Although there is a difference in the melting peaks – there is a peak observed at 58°C when performed on the BioRad CFX96^{TM} Real-Time PCR Detection System (bottom image) but not on the LC480 (top image) – there was no statistical difference ($P<0.05$) between the data performed on the two machines.

Appendix A3: **pA259 [pGEM-T easy::17α-CYP6P9 (FANG), with no Histidine tag]**

GGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAG
GAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGT
AACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCATTCGCCATTCAGGCTGCGC
AACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGT
GCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCC
AGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCAT
GGCCGCGGGATTTTTCATATGGCTCTGTTATTAGCAGTTTTTGCCGCGTTCATCTTCGTAGTGTCGGC
AGTGTACCTTTTCATTCGGAACAAACATAATTACTGGAAAGACGATGGATTCCCGTATGCGCCGAA
CCCACATTTTCTGTTCGGACACGCGAAAGGACAGGCCCAGACAAGGCATGCGGCCGACATCCATCT
GGAACTGTACAAAAAATTCAAGCAGCGCCGTGAGCGGTACGTTGGTGTGAGCCAGTTCATGATACC
TTCATTGCTCGTGATCGATCCAGAGCTGGTGAAAACGATCCTAGTAAAGGACTTTAATGTATTCCAC
GATCATGGTGTATTCAATAATGCAAGAGACGACCCGCTGTCCGCACATCTTTTTGCGCTTGAAGGTA
ACCCATGGCGCTTGTTGCGTCAGAAGCTCACGCCAACGTTCACCTCAGGTCGCATGAAGCAAATGT
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TCGAGAAAGTAACAACATTAAACGAAACGACTTCATGAACCTGCTGTTGCAGATTAAGAATAAGGG
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CGAGGAGAATGACGGCCAGGTGACGTACGATGTCGCCATAAACATACAGTATCTGGACAATGTGAT
AAACGAAACACTTCGCAAGTACCCACCGGTAGAATCGTTGAGTCGTGTGCCGTCTGTTGACTATGTC
ATCCCAGGTACGAAACATGTCATTCCCAAACGAACGTTAGTGCAAATTCCGGTTCACGCCATTCAA
CATGATCCTGAGCACTGTCCCGATCCAGAACGTTTCGACCCGGATCGCTTCTCACCGGAGGAAGTG
AAGAAGCGACATCCCTTCACGTTCCTCCCATTCGGTGAGGGGCCACGCGTTTGCATTGGGCTTCGGT
TTGGTGTGATGCAGACGAAGGTAGGATTGATAACGCTGTTGAGAAAGTTCCGCTTCTCACCGTCAG
CGCGTACACCAGATTGTGTAAAGTTCGATCCGAAAATGATCATTCTGTCACCGATCGCGGGTAATTA
CTTGAAGGTGGAAAAGTTGTAGTCTAGAAAAATCACTAGTGCGGCCGCCTGCAGGTCGACCATAG
GGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGT
AATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGC
CGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCG
CTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCG
GGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCG
TTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGA
ATAACGCAGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAGGCC

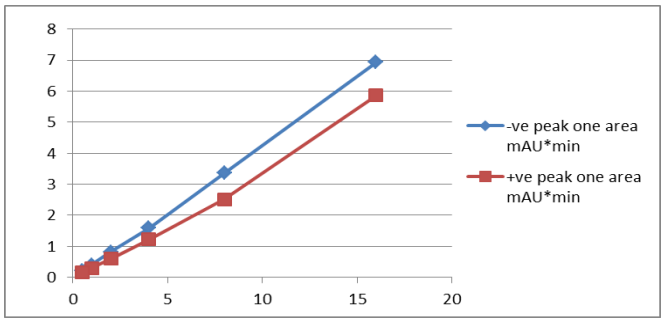
PURPLE = 17α hydroxylase sequence (MALLLAVF) with

GREEN = NdeI restriction site

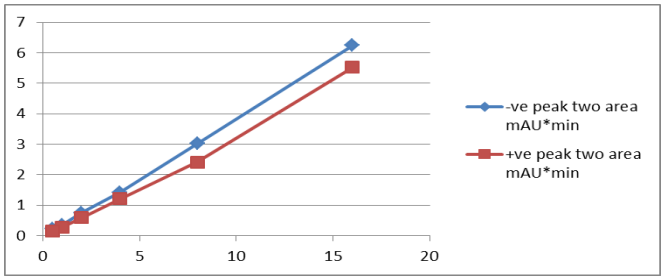
BLUE = XbaI restriction site

APPENDIX A4 – **Kinetic raw data and calculations, graphs and plots**

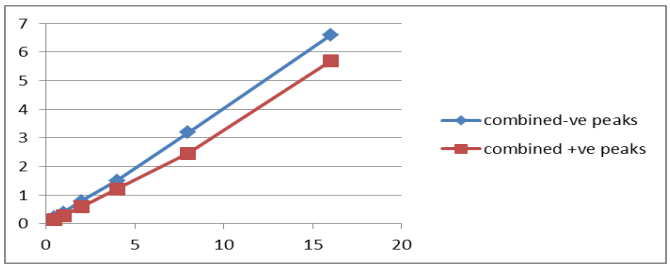
concentration (uM)	-ve peak one area mAU*min	+ve peak one area mAU*min
16	6.9228	5.8531
8	3.3554	2.5063
4	1.5817	1.2167
2	0.8157	0.5937
1	0.4026	0.2881
0.5	0.2105	0.1436



concentration (uM)	-ve peak two area mAU*min	+ve peak two area mAU*min
16	6.2474	5.5257
8	3.022	2.4074
4	1.4269	1.2107
2	0.7558	0.5917
1	0.361	0.2818
0.5	0.2222	0.1441

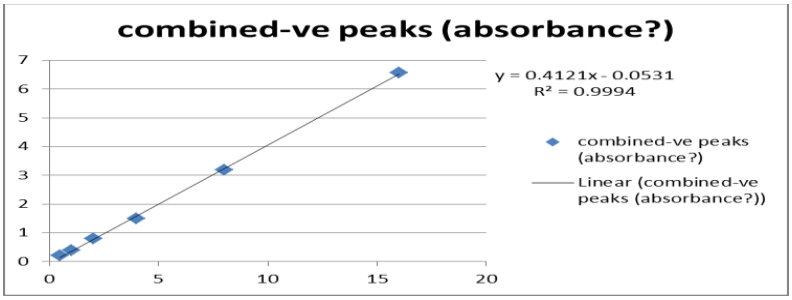


concentration (uM)	Combined –ve peaks	combined +ve peaks
16	6.5851	5.6894
8	3.1887	2.45685
4	1.5043	1.2137
2	0.78575	0.5927
1	0.3818	0.28495
0.5	0.21635	0.14385

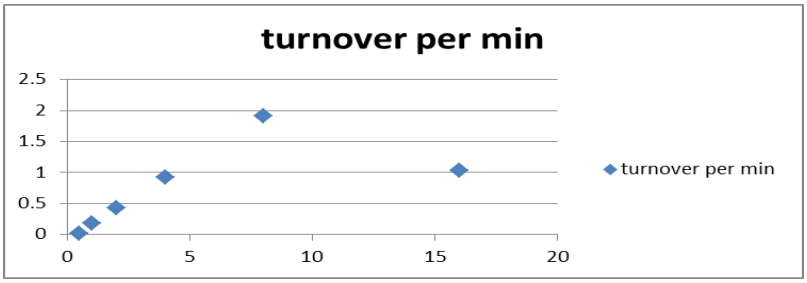


concentration (uM)	difference	turnover per min
16	13.93472	2.065275
8	6.090633	1.909367
4	3.074011	0.925989
2	1.567095	0.432905
1	0.820311	0.179689
0.5	0.477918	

concentration (uM)	Combined -ve peaks
16	6.5851
8	3.1887
4	1.5043
2	0.78575
1	0.3818
0.5	0.21635

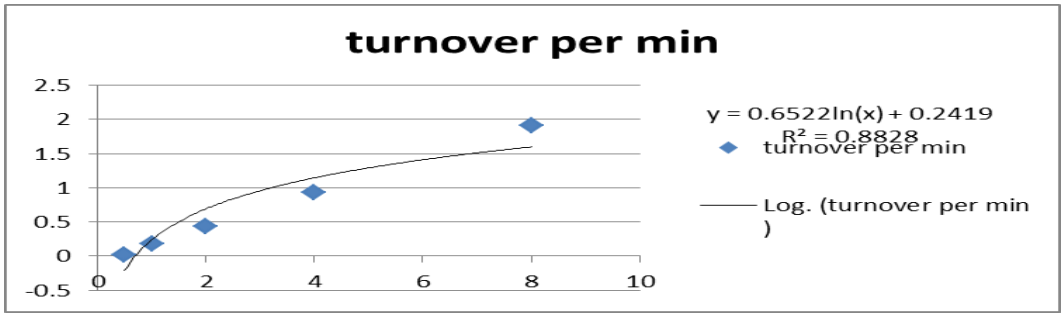


concentration (uM)	turnover per min
16	1.032638

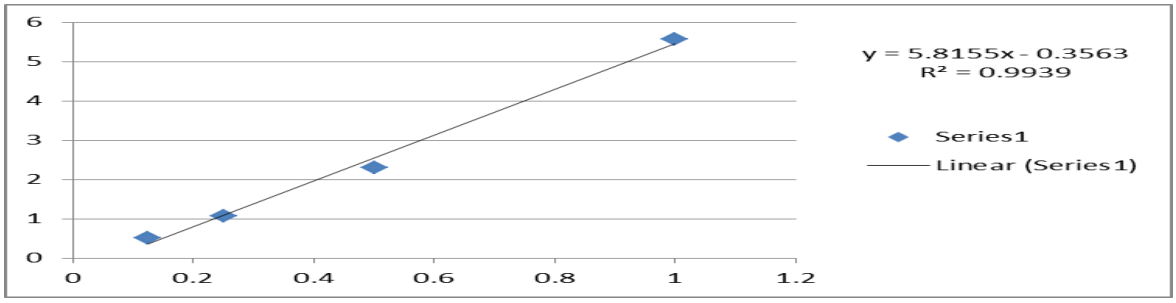


8	1.909367
4	0.925989
2	0.432905
1	0.179689
0.5	0.022082

Excluding 16uM



Reciprocal plot (i.e. 1/concentration versus 1/turnover per min)



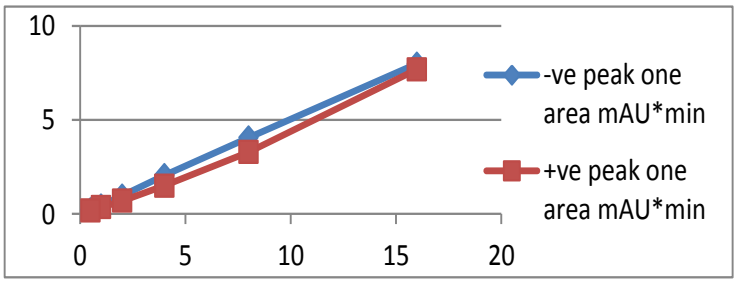
2.806624	**Vmax**	= (1/0.3563)
0.061267		(=0.3563/5.8155)
16.32192	**Km (uM)**	(= 1/0.061267)

Calculated using sigmaPlot 11:

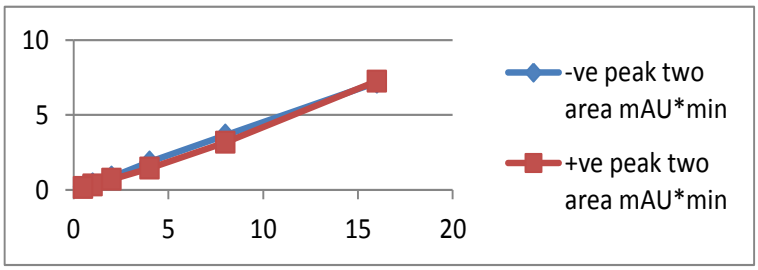
Parameter	Value	StdErr	Dependencies
a (Vmax)	**2.28**	0.25	0.950839
b (Km)	**6.90**	1.30	0.950839

Second run

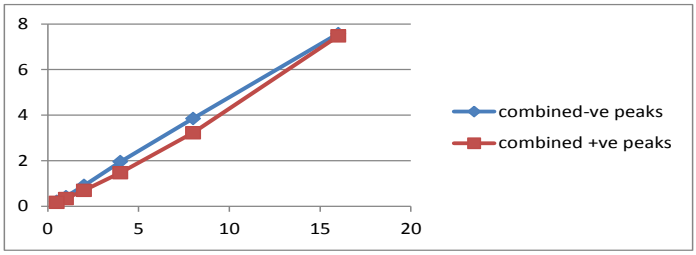
concentration (uM)	-ve peak one area mAU*min	+ve peak one area mAU*min
16	7.9937	7.6858
8	4.054	3.288
4	2.056	1.5012
2	0.9712	0.7007
1	0.453	0.3539
0.5	0.2402	0.1833



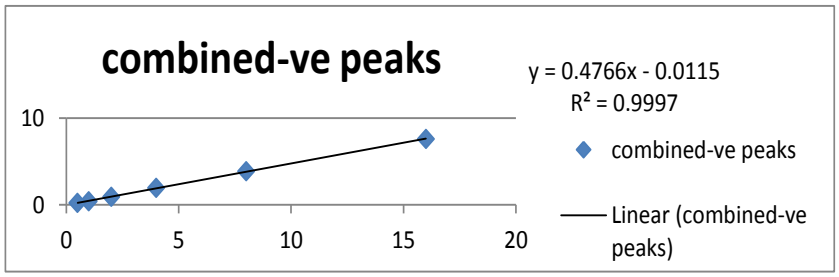
concentration (uM)	-ve peak two area mAU*min	+ve peak two area mAU*min
16	7.1672	7.255
8	3.647	3.1634
4	1.8616	1.4482
2	0.8598	0.695
1	0.3917	0.3273
0.5	0.1941	0.1586



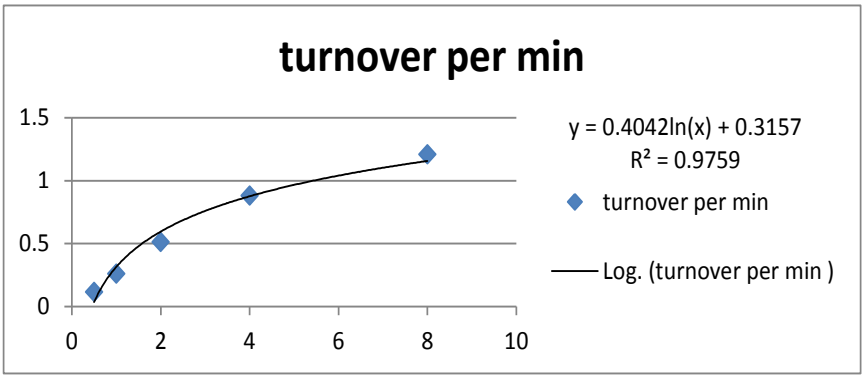
concentration (uM)	combined-ve peaks	combined +ve peaks
16	7.58045	7.4704
8	3.8505	3.2257
4	1.9588	1.4747
2	0.9155	0.69785
1	0.42235	0.3406
0.5	0.21715	0.17095

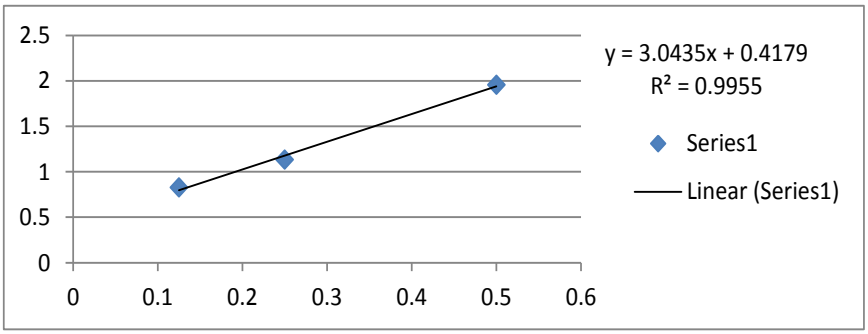


concentration (uM)	Combined -ve peaks
16	7.58045
8	3.8505
4	1.9588
2	0.9155
1	0.42235
0.5	0.21715



concentration (uM)	turnover per min
16	0.150755
8	1.207721
4	0.881662
2	0.511645
1	0.261225
0.5	0.117184





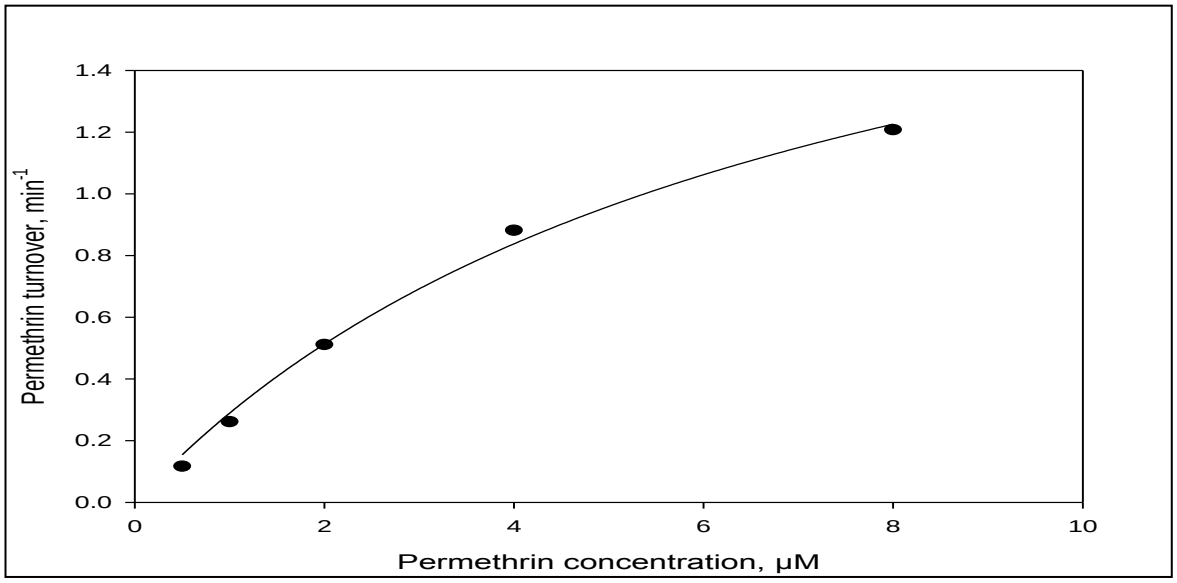
2.392917 Vmax

0.137309

7.282843 Km (uM)

Calculated with sigmaPlot 11:

Parameter	Value	StdErr	CV(%)	Dependencies
a (Vmax)	**2.28**	0.25	10.76	0.950839
b (Km)	**6.90**	1.30	18.77	0.950839



SIGMA PLOT 11 DATA:

	1-concentration	2-rate
8	1.2077	0.5
4	0.8817	0.5293
2	0.5116	0.5586
1	0.2612	0.5879
0.5	0.1172	0.6172

Nonlinear Regression
Data Source: Data 1 in Notebook1
Equation: Hyperbola, Single Rectangular, 2 Parameter
f=a*x/(b+x)

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9973	0.9945	0.9927	0.0383

	Coefficient	Std. Error	t	P
a	2.2823	0.2456	9.2941	0.0026
b	6.8953	1.2945	5.3265	0.0129

Analysis of Variance:

	DF	SS	MS
Regression	2	2.5753	1.2876
Residual	3	0.0044	0.0015
Total	5	2.5797	0.5159

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	0.7999	0.7999	545.7456	0.0002
Residual	3	0.0044	0.0015		
Total	4	0.8043	0.2011		

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.3531)
W Statistic= 0.8892 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.0500)

Fit Equation Description:
[Variables] x = col(1); y = col(2)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2
[Parameters] a = max(y) "Auto {{previous: 2.28228}}; b = x50(x,y,0.1) "Auto {{previous: 6.89532}}
[Equation] f=a*x/(b+x); fit f to y; "fit f to y with weight reciprocal_y; "fit f to y with weight reciprocal_ysquare
[Constraints] [Options] tolerance=1e-10 ; stepsize=1; iterations=200

Number of Iterations Performed = 9

Nonlinear Regression
Data Source: Data 1 in Notebook1
Equation: Hyperbola, Single Rectangular, 2 Parameter
f=a*x/(b+x)

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9973	0.9945	0.9927	0.0383

	Coefficient	Std. Error	t	P
a	2.2823	0.2456	9.2941	0.0026
b	6.8953	1.2945	5.3265	0.0129

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Regression	2	2.5753	1.2876
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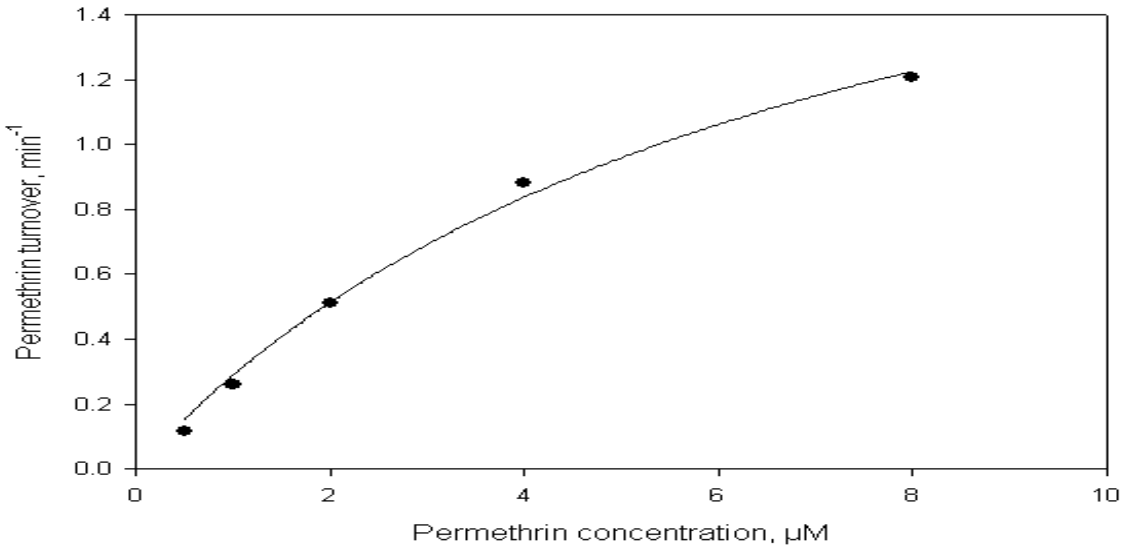
Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	0.7999	0.7999	545.7456	0.0002
Residual	3	0.0044	0.0015		
Total	4	0.8043	0.2011		

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.3531)
W Statistic= 0.8892 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.0500)

Fit Equation Description:
[Variables] x = col(1); y = col(2); reciprocal_y = 1/abs(y); reciprocal_ysquare = 1/y^2
[Parameters] ; a = max(y) "Auto {{previous: 2.28228}} ; b = x50(x,y,0.1) "Auto {{previous: 6.89532}}
[Equation] f=a*x/(b+x) ; fit f to y; "fit f to y with weight reciprocal_y ; "fit f to y with weight reciprocal_ysquare
[Constraints] [Options] ; tolerance=1e-10; stepsize=1 ; iterations = 200
Number of Iterations Performed = 9



Data 2:

16	1.0326	0.5	0.2025
8	1.9094	0.5605	0.224
4	0.926	0.6211	0.245
2	0.4329	0.6816	0.2654
1	0.1797	0.7422	0.2853

Nonlinear Regression
Data Source: Data 2 in 10Aug_results.JNB
Equation: Hyperbola, Single Rectangular, 2 Parameter
f=a*x/(b+x)

R	**Rsqr**	**Adj Rsqr**	**Standard Error of Estimate**
0.8274	0.6846	0.6058	0.4360

	Coefficient	**Std. Error**	**t**	**P**
a	1.8390	0.8165	2.2523	0.0874
b	4.0417	4.6439	0.8703	0.4332

Analysis of Variance:

	DF	**SS**	**MS**
Regression	2	5.0293	2.5147
Residual	4	0.7603	0.1901
Total	6	5.7897	0.9649

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	1.6503	1.6503	8.6824	0.0421
Residual	4	0.7603	0.1901		
Total	5	2.4107	0.4821		

Statistical Tests:

Normality Test (Shapiro-Wilk) Passed (P = 0.0708)

W Statistic= 0.8091 Significance Level = 0.0500

Constant Variance Test Passed (P = 0.0600)

Fit Equation Description:

[Variables]; x = col(1); y = col(2)

reciprocal_y = 1/abs(y)

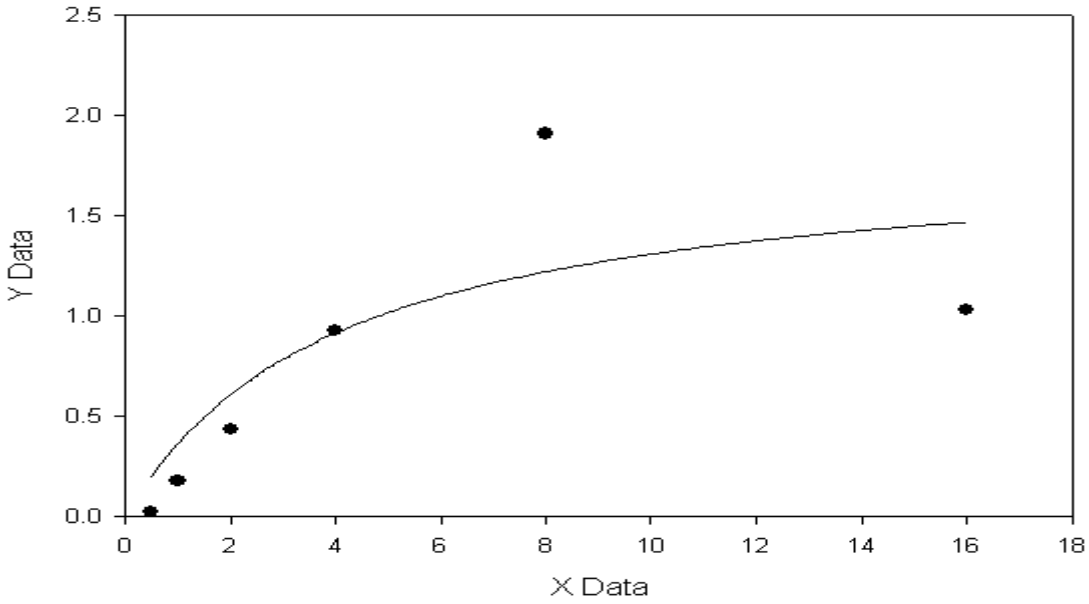
reciprocal_ysquare = 1/y^2

[Parameters]; a = max(y) "Auto {{previous: 1.83897}}; b = x50(x,y,0.1) "Auto {{previous: 4.04169}}

[Equation] ; f=a*x/(b+x) ; fit f to y ; "fit f to y with weight reciprocal_y ;"fit f to y with weight reciprocal_ysquare

[Constraints] [Options] ; tolerance=1e-10 ; stepsize=1; iterations=200

Number of Iterations Performed = 9



Appendix A5: **Peptide synthesis and production of monoclonal antibody CYP6P9**

A) 2D-Electrophoresis

I. *First dimension: Isoelectric focusing (IEF)*

- Samples (proteins expressed in tested cell lines or purified proteins) were prepared in Urea lysis buffer (*Appendix A5 – 2D ELFO solutions*) and run in IEF on an Immobiline DryStrip, pH 3-10 NL (Amersham-Pharmacia-Biotech).
- Sample (30µl; 100µg of total protein concentration per DryStrip) plus 95µl of reswelling buffer (*Appendix A5 – 2D ELFO solutions*) was loaded and the DryStrip was covered with mineral oil and incubated overnight at room temperature (RT).
- IEF was run under the following conditions: 6min/200V, 100min/1750V, 60min/3500V.

II. Second dimension: SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

- The Immobiline DryStrip was equilibrated in SDS equilibration buffer (*Appendix A5 – 2D ELFO solutions*) with addition of 2% DTT:
- For 15 minutes and with addition of 2.5% iodoacetamide and 0.01% bromophenol blue
- For another 7 minutes (*Appendix A5 – 2D ELFO solutions)*
- The proteins from IEF were separated by SDS-PAGE using 7.5%-15% gel. Strips were loaded using 0.5% agarose dissolved in SDS-PAGE running buffer. Separated proteins were transferred onto nitrocellulose membrane in either Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell for 2hrs (4°C, 150mA) in transfer buffer or in Trans-Blot SD Semi-Dry Transfer-Cell unit (BioRad) for 1hr (RT, 200mA) using transfer buffer.
- The pre-stained molecular weight markers (Bio-Rad) were run in parallel.

2D ELFO solutions:

Lysis buffer:	**25ml**
9 M Urea	13.5135g
2% NP-40	500µl
3% CHAPS	750µl
70 mM DTT	270mg

Before use: Aprotinin 1 µg/ml

Leupeptin 1 µg/ml	Protease inhibitor kit (Roche)
2 mM Na_3VO_4	
1mM PMSF	200µl
25mM NaF	26.24mg
2% Ampholyte pH 9-11	20 µl/1ml buffer

Reswelling buffer for IEF strips:	**25 ml**
7M Urea	10.5105g
0.5% Triton X-100	125µl
10mM DTT	38.55mg
2M Thiourea	3.806g
4% CHAPS	1g
0.003% Bromphenol Blue	150µl (0.5%)
Add before use: 1 % Ampholyte pH 3-10	*10 µl/1ml buffer*

SDS Equilibration buffer after IEF (5 ml for 1 strip):	**100ml**
50mM Tris, pH 6.8	5ml (1M)
6M Urea	36g
30% Glycerol	30ml
2% SDS	2g

Incubations:

A. Add 2% DTT (0.2g/10ml) and incubate 15min/RT.

B) Add 2.5% iodoacetamide (0.25g/10ml) and 0.01% bromophenol blue (200µl, 0.5%/10ml) and incubate 7min/RT.

SDS stock solution:	**500ml**
20% SDS	100g

Acrylamide mix (30%):	**100ml**
30% Acrylamide	30g
0.8% Bis-acrylamide	0.8g

Tris Buffers:	**100ml**
1M Tris-HCl, pH 8.8	12.1g
1M Tris-HCl, pH 6.8	12.1g

Running gel (30ml):	**7.5%**	**10%**	**12.5%**	**15%**
Acrylamide mix (30%)	7.5ml	10ml	12.5ml	15ml
1M Tris-HCl pH 8.8	11.2ml	11.2ml	11.2ml	11.2ml
20% SDS	0.15ml	0.15ml	0.15ml	0.15ml
Deionized H_20	13.7ml	11.2ml	8.7ml	3.7ml

Add before use: 80µl of 10% amonium persulfate (APS) and 20µl of TEMED

Stacking gel (30ml):	**5%**	**3%**
Acrylamide mix (30%)	1.67ml	1.0ml
1 M Tris-HCl pH 6.8	1.25ml	1.25ml
20% SDS	0.05ml	0.05ml
Deionized H_2O	7.03ml	7.7ml

Add before use: 50µl of 10% amonium persulfate (APS) and 10µl of TEMED

B) ELISA assay (Two captured assay)

- A 96-well microtitre plate (Becton Dickinson - FALCON 3912) was incubated overnight at RT with 50µl per well of 10-20µg/ml Ig of purified antibody (monoclonal or polyclonal) diluted in buffer E1 (*Appendix A5 – ELISA buffers*).
- The plate was rinsed in PBS (1X) and blocked for 2hrs in 3% bovine serum albumin (BSA, SIGMA, A-7030) in PBS (200µl per well, RT).
- A washing step: PBS (1X), PBS + 0.1% NP-40 (2X), PBS (1X) followed. The extract (antigen) was added – 50µl of serial two-fold dilutions either in lysis buffer or PBS per well (minimal total protein concentration 1 mg/ml) and allowed to incubate for 2hrs at 4ºC.
- The washing step: PBS (1X), PBS + 0.1% NP-40 (2X), PBS (1X) followed.
- Antibody was added (50µl of 1µg/ml of antibody per well) (if coated by monoclonal add polyclonal; if coated by polyclonal add monoclonal) diluted in 1% BSA in PBS and incubated for 2hrs at 4ºC.
- The washing step as before then followed.
- Peroxidase-conjugated swine antiserum was added to rabbit immunoglobulin or rabbit antiserum was added to mouse immunoglobulin (DAKO), diluted 1:1000 in 1% BSA in PBS; 50µl per well. This was left to incubate for 1hr at 4ºC, followed by the wash step as before.
- Visualization was carried out with tetramethylbenzidine: 6ml of buffer E2 (*Appendix A5 – ELISA buffers*) + 60µl of solution E3 (*Appendix A5 – ELISA buffers*) + 15µl of H_2O_2 - aliquot 50µl per well. This was left for 20-30 min and the reaction stopped with 1M H_2SO_4 (50µl per well).
- The results were monitored – O.D. 450nm.

ELISA Buffers

Buffer E1: Buffer for coating plates (340ml):
0.1M CO_3/HCO_3 pH 9-9.8
0.42g Na_2CO_3 (SIGMA)
2.52g $NaHCO_3$ (SIGMA)

Buffer E2: Substrate buffer; 0.1M citrate/phosphate, pH 6.0 (650ml):
7.05g Na_2HPO_4 (SIGMA)
3.15g citric acid (SIGMA)

Buffer E3: Substrate: 3,3', 5,5'– tetramethylbenzidine (TMB: SIGMA – T-2885)
10mg/ml TMB in DMSO (SIGMA)

C) <u>Immunization of the mice</u>

Mice BALB/C was used for the immunization protocol.

For each protein 5 mice were used.

Immunization protocol*:*

1) 1st injection – ***day 1***: intraperitoneal injection of 10–50µg of purified protein or peptide coupled to KLH (in complete Freund's adjuvants).
2) 2nd injection – ***day 21***: intraperitoneal injection of 10–50µg of purified protein or peptide coupled to KLH (in incomplete Freund's adjuvants).
3) 3rd injection – ***day 42***: same as day 21
4) 4th injection – ***day 45***: intraperitoneal injection of 10µg of purified protein or peptide coupled to KLH (in PBS).
5) 5th injection – ***day 46***: same as day 45
6) Fusion – ***day 47*** *(production of monoclonal antibodies)*

D) Affinity purification of mouse sera on peptide used as immunogen

Day 1: Pierce 6x reactigel beads (Cat. No: 20259) (0.5ml) were washed with 0.1M Sodium Borate buffer (pH 9.0) in an eppendorf tube (to exchange the acetone). Peptide (5mg or 1mg - minimum) was added to the beads and borate buffer was added to make up a final volume of 1.5ml. The peptide and beads were allowed to mix overnight in a wheel at RT.

Day 2: The beads were spun down, supernatant removed and 1ml of 1M Tris HCl pH 8.8 was added and allowed to mix throughout the day. Residual peptide was washed away very well [about 10-15 washes in PBS-Tween-20 (0.1%) depending upon the volume of each wash] and the beads (containing 1-5mg of coupled peptide) were added to a 50ml tube containing up to 50ml of serum. This was allowed to mix overnight.

Day 3: The beads were spun and serum saved. Serum proteins were washed away with PBS-Tween-20 (0.1%) and the beads were transferred to an eppendorf tube and 1ml of 0.1M Glycine (pH 2.5) was then added to it to elute the IgG. Beads were mixed in 0.1M Glycine (pH 2.5) for 30min. The supernatant (1ml) was removed and 0.15ml of 1.5M Tris-HCl pH 8.8 was added to it to neutralise the acid. Another 1ml of 0.1M Glycine (pH 2.5) was added to the beads and mixed for 30min, and this step was repeated another three times. A 10% or 12% SDS gel with 10µl of each fraction was run to see which fractions the IgG came off in (generally fractions 1, 2, and 3). The Ig concentration was measured using Bio-Rad Protein Assay (to do standard curve use bovine gamma globulin).

Note: The beads can be reused - *Reapply the 1x used serum to the beads and repeat the purification steps to see if you adsorbed all the IgG from the serum.*

E) Mass spec (MS) and HPLC analysis for sample CVK 70301 (CVKFDPKMIIL)

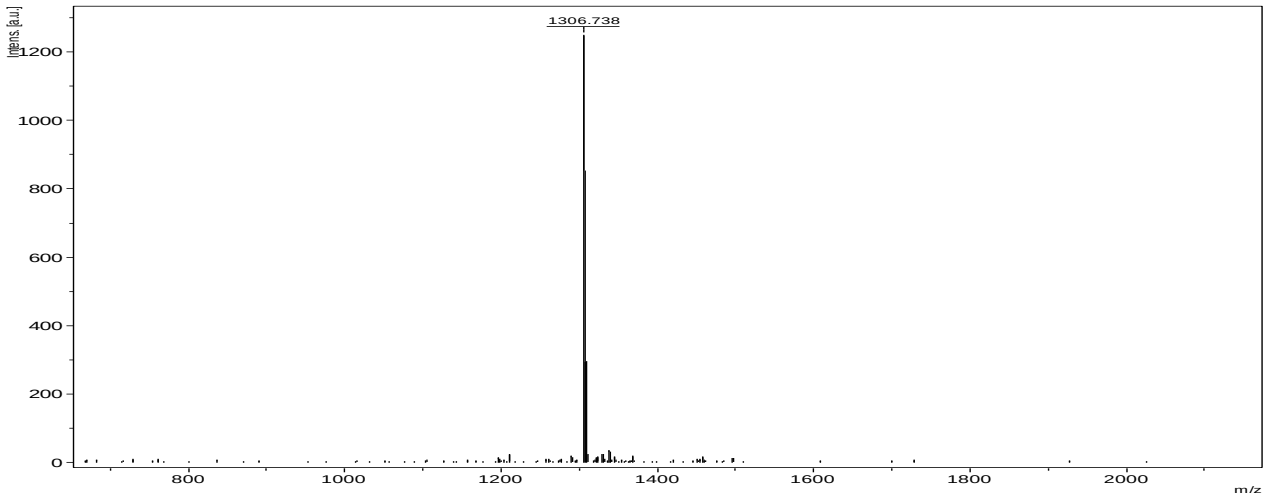


Figure E-1: MALDI-TOF MS analysis report showing purity of sample CVK 70301 (Sequence CVKFDPKMIIL) >86%. Y-axis = atomic mass units (amu); X-axis – mass/charge ratio (m/z). MH+ (theoretical) = 1306.738. In MS analysis, ions generally have a single charge, therefore the m/z value is equivalent to mass itself. Each bar represents an ion having a specific m/z ratio and the length of the bar indicates the relative abundance of the ion. The highest mass = the molecular ion; the lower mass ions = fragments from the molecular ion assuming the sample is a single compound.

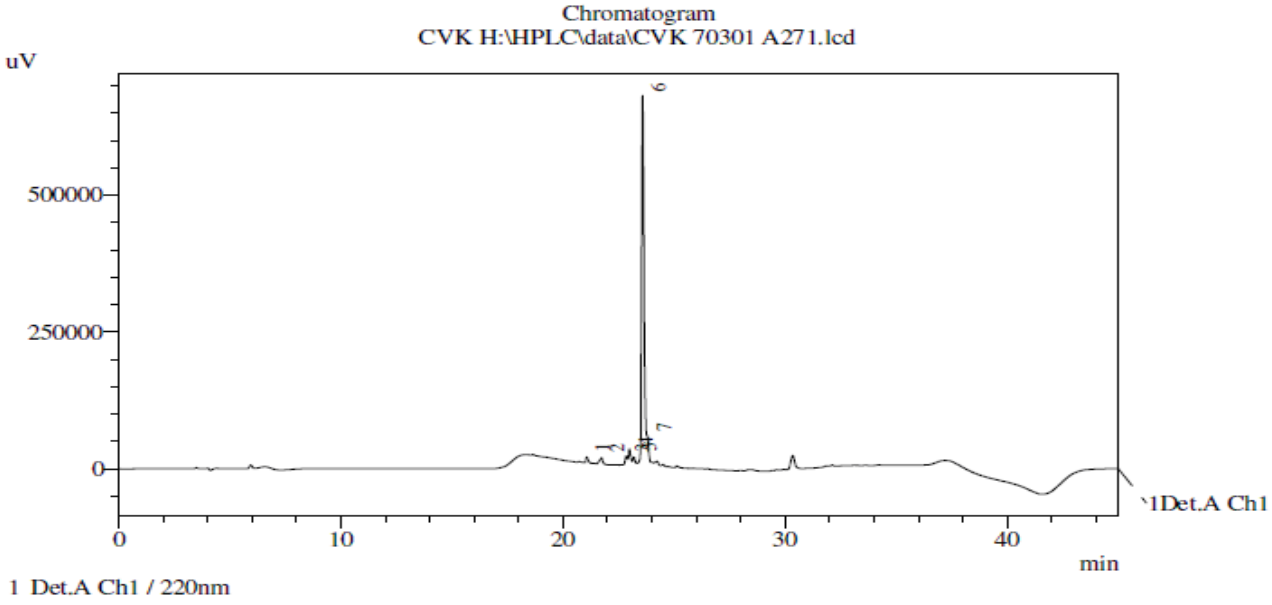


Figure E-2: HPLC Analysis of sample CVK 70301. Injection volume was 100µl, with UV detection at Ch1/220nm. Sample was run over a time period of 40min. Peak 6 showed a retention time of 23.585min, Area = 5093407, height = 662588, Area % = 86.001 and height % = 83.843.

Appendix A6: **Immunizations and dot blots**

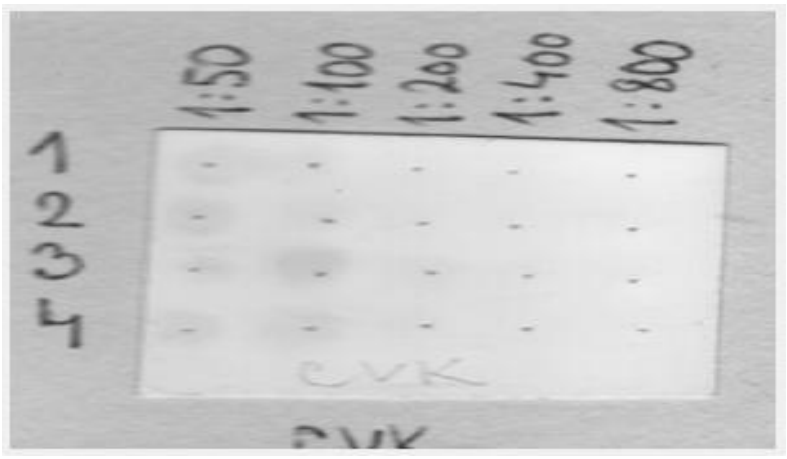


Figure A6-1: Mouse sera to CVK after 2nd immunization dot blot showing mouse number versus dilution to CVK.



Figure A6-2: Mouse sera to CVK after 3rd immunization dot blot showing mouse number versus dilution to CVK.

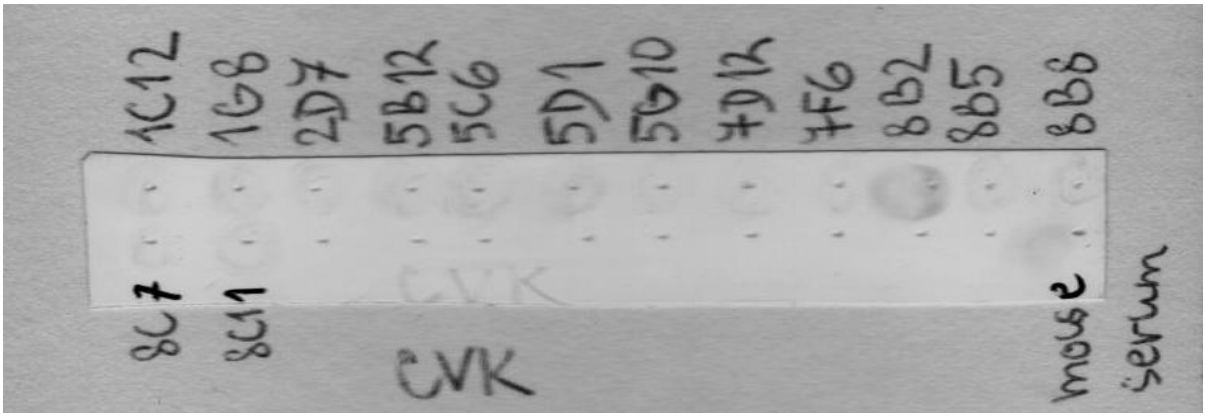
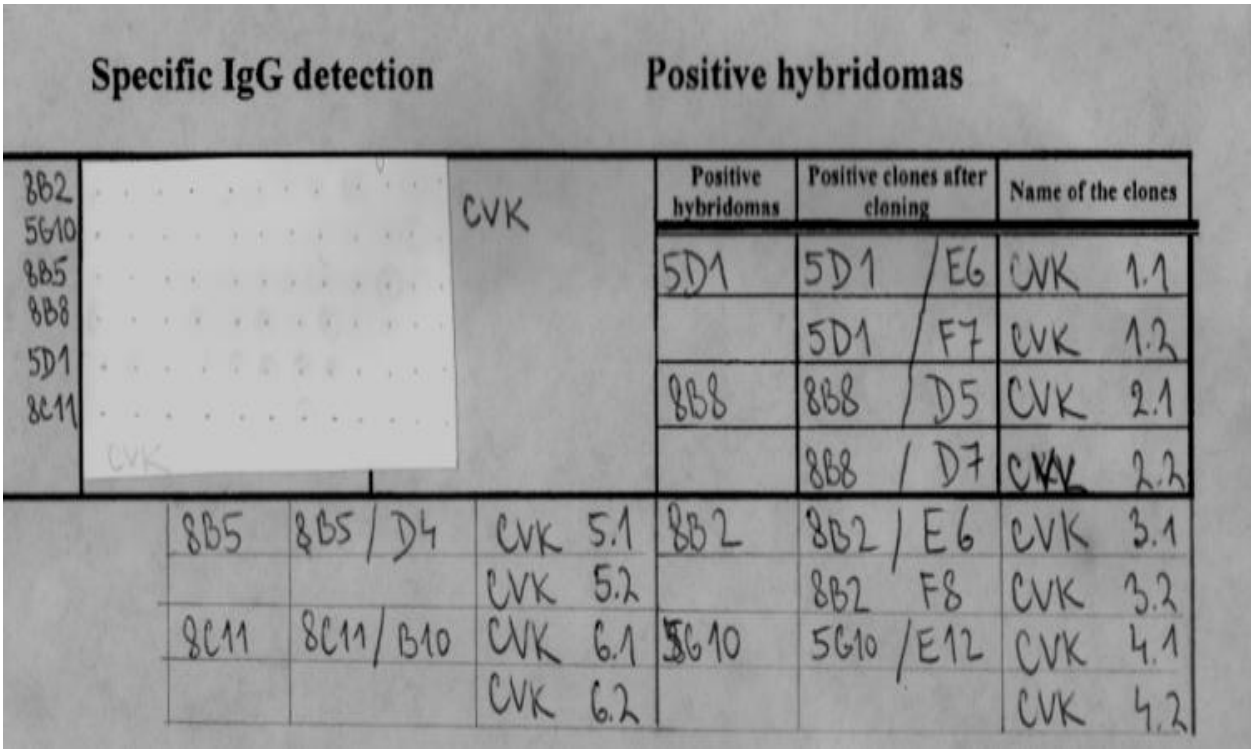


Figure A6-3: Supernatants of fusion CVK dot blot. 8B2 shows the strongest fusion reaction. 5D1, 5G10, 8B2, 8B5, 8B8 and 8C11 were selected for use in the final hybridoma analysis.



Specific IgG detection **Positive hybridomas**

8B2 5G10 8B5 8B8 5D1 8C11	CVK		Positive hybridomas	Positive clones after cloning	Name of the clones
			5D1	5D1 / E6	CVK 1.1
				5D1 / F7	CVK 1.2
			8B8	8B8 / D5	CVK 2.1
				8B8 / D7	CVK 2.2
8B5	8B5 / D4	CVK 5.1	8B2	8B2 / E6	CVK 3.1
		CVK 5.2		8B2 F8	CVK 3.2
8C11	8C11 / B10	CVK 6.1	5G10	5G10 / E12	CVK 4.1
		CVK 6.2			CVK 4.2

Figure A6-4: CVK 3.1 from hybridoma 8B2/E6 was the only positive hybridoma for IgG1, Kappa. The others (CVK 1.1, CVK 2.1, CVK 4.1, CVK 5.1 and CVK 6.1 are all IgM, Kappa).

Appendix A7: **Other buffers and reagents used in this study**

10X MOPS running buffer (RNA gel)

0.4M MOPS

0.1M sodium acetate

0.01M EDTA (disodium)

Adjust pH to 7.0 using NaOH or Glacial Acetic acid CH_3COOH

10X loading RNA buffer Recipe

0.35% (w/v) Orange G

30% (w/v) Ficoll 400

1mM EDTA (disodium)

For electrophoresis buffer: dilute 10X running buffer to make 1X running buffer

RNA agarose gel (1%, 20ml), prepared with 1X running buffer and 7% formaldehyde:

0.2g agarose

2ml 10X running buffer

14.2ml ultrapure water

Microwave for 45 seconds; then allow to cool. Add 3.8ml of 37% formaldehyde and then pour into casting tray with combs.

To prepare RNA for loading onto wells combine the following:

3.5 µl of each RNA sample with:

10 µl formamide

3.5 µl 37 % formaldehyde

1 µl 10X running buffer

2 µl 10X loading buffer

Denature RNA in buffers for 10 minutes t 65-75 °C. Then load into gel wells.

LB broth for ligations and transformations:

Liquid broth

2.5 g Bacto-Tryptone

1.25 g Bacto-yeast extract

1.25 g NaCl

Make up to 250 ml with dH2O

For Solid Broth, mix the same as for Liquid Broth, but include 3.75g Agar (1.5%) before making up with dH2O.

Liquid and solid broths are then autoclaved, allowed to cool and then:

To the Solid broth, 100 µg/ml Ampicillin is added, and

To the Liquid broth 50 µg/ml Ampicillin is added.

SDS-PAGE (Laemmli) Buffer system

Stock solutions and buffers

1. **Bis/Acrylamide** (30% T, 2.67% C)

2. **10% (w/v) SDS**

   Dissolve 10g SDS in 90ml watetr with gentle stirring and bring to 100ml with deionized water. Alternatively, 10% SDS solution (250ml) can be used (Bio-Rad cat no: 161-0416).

3. **1.5M Tris-HCl, pH 8.8 (resolving gel buffer)**

   27.23g Tris base (18.15g/100ml)

   80ml deionised water

   Adjust to pH 8.8 with 6N HCl. Bring total volume to 150ml with deionized water and store at 4°C.

4. **0.5M Tris-HCl, pH 6.8 (Stacking gel buffer)**

6g Tris base

50ml deionised water

Adjust to pH 6.8 with 6N HCl. Bring total volume to 150ml with deionized water and store at 4°C.

5. **Laemmli 2x sample buffer (mixed 1:1)**

3.55ml deionized water

1.25ml 0.5M Tris-HCl, pH 6.8

2.5ml glycerol

2.0ml 10% (w/v) SDS

0.2ml 0.5% (w/v) bromophenol blue

9.5ml total volume

Store at room temperature

Before use: add 50µl β-mercaptoethanol to 950µl sample buffer. Dilute the sample at least 1:2 with sample buffer and heat at 95°C for 4 minutes.

6. **10X Electrode (Running) buffer, pH 8.3 (makes 1L)**

30.3g Tris-base

144.0g glycine

10.0g SDS

Dissolve and bring total volume up to 1L with deionized water. Do not adjust pH with acid or base. Store at 4°C.

Before use: dilute 50ml of 10X stock with 450ml deionized water for each electrophoresis run. Mix thoroughly.

7. **10% (w/v) APS (fresh daily)**

100mg ammonium persulphate

Dissolve in 1ml of deionized water.

8. **Gel formulations (10ml)**

a. Prepare the monomer solution by mixing all reagents except the TEMED and 10%APS. Degas the mixture for 15 minutes.

Percent gel	DDI H_2O (ml)	30% Degassed Acrylamide/Bis (ml)	Gel buffer * (ml)	10% w/v SDS (ml)
5%	5.7	1.7	2.5	0.1
12%	3.4	4.0	2.5	0.1
15%	2.4	5.0	2.5	0.1

* Resolving gel buffer – 1.5M Tris-HCl, pH 6.8

* Stacking gel buffer – 0.5M Tris-HCl, pH 8.8

b. Immediately prior to pouring gel, add: (*For 10ml monomer solution*)

Resolving gel: 50µl 10% APS and 5µl TEMED

Stacking gel: 50µl 10% APS and 10µl TEMED

9. **Transfer buffer (1L)**

25mM Tris-base

190mM glycine

20% methanol

Store at 4°C

10. **Coomassie Stain (1L)**

1g Coomassie R250

100ml glacial acetic acid

400ml methanol

500ml ddH_2O

Store at room temperature in a sealable container

11. **De-Stain for Coomassie (1L)**

200ml methanol

100ml glacial acetic acid

700ml ddH_2O