Supplementary Information

The combination of vemurafenib and procaspase-3 activation is synergistic in mutant BRAF melanomas

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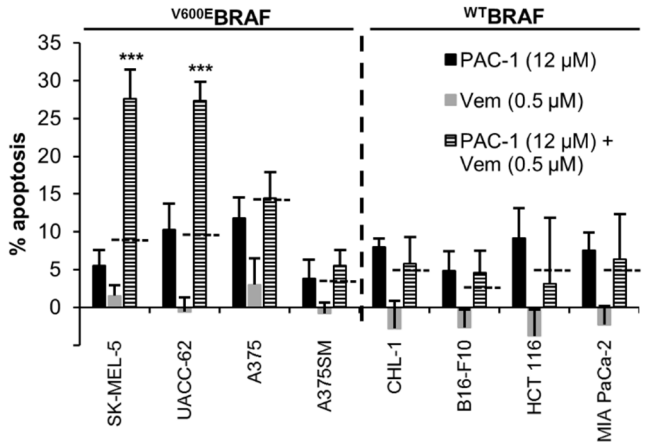
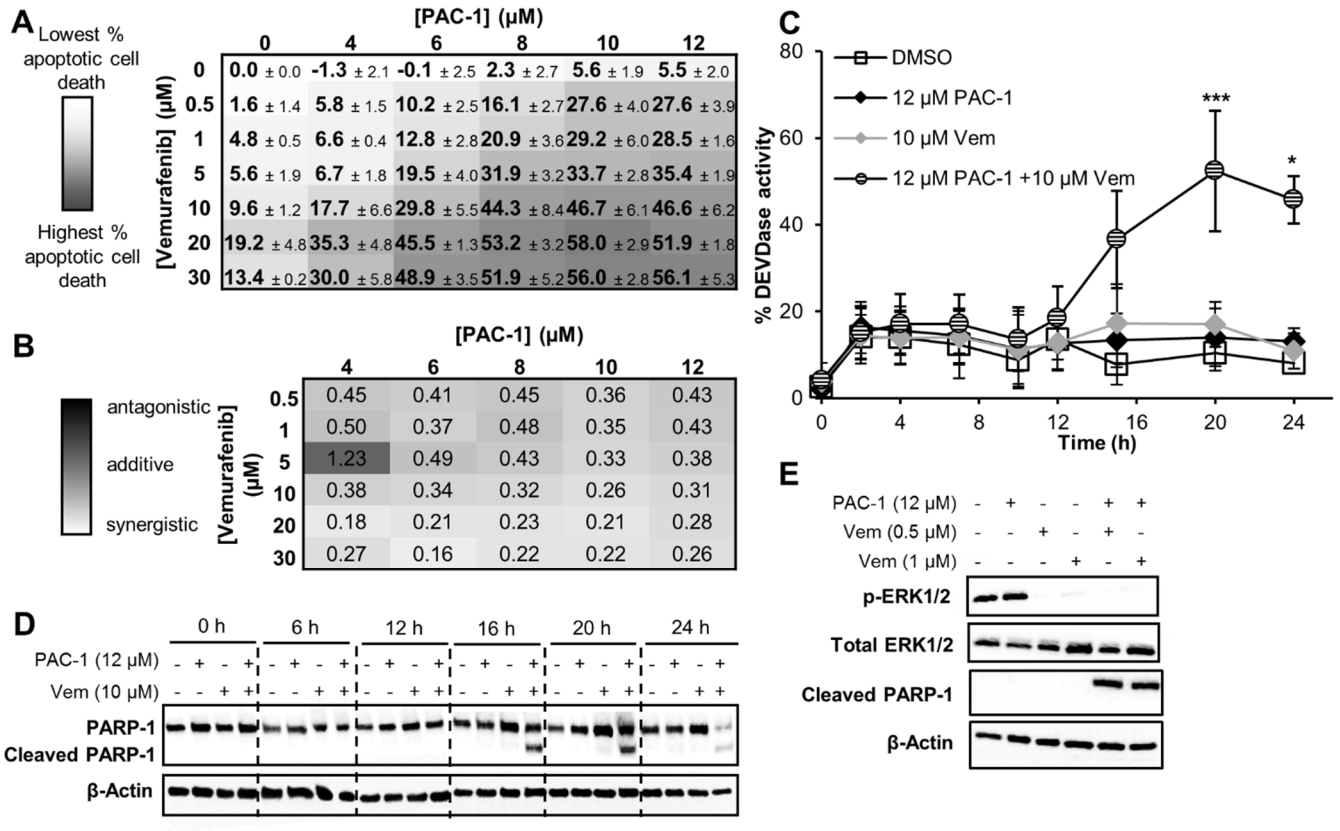
Primer sequences used to characterize vemurafenib-resistant A375VR

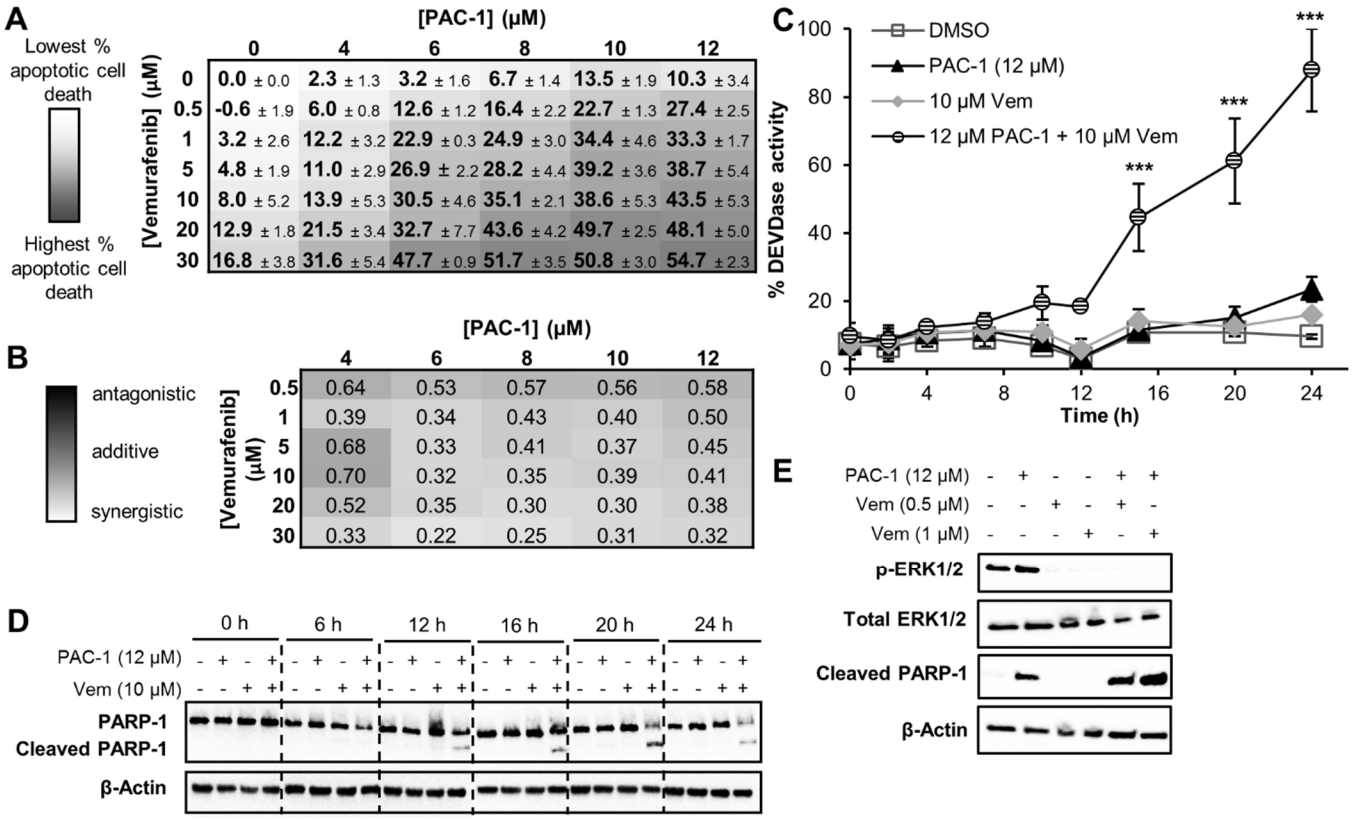
MDR1 F	ACACCATGGGGAAGGTGAAG
MDR1 R	GTGACCAGGCGCCCAATA
GAPDH F	ACACCATGGGGAAGGTGAAG
GAPDH R	GTGACCAGGCGCCCAATA
BRAF F	GGCTCTCGGTTATAAGATGGC
BRAF R	ACAGGAAACGCACCATATCC
MEK1 Amp F	CGTTACCCGGGTCCAAAATG
MEK1 Amp R	CTTTGTCACAGGTGAAATGC
MEK1 Seq F	CATGGATGGAGGTTCTCTGG
MEK1 Seq R	AGGGCTTGACATCTCTGTGC
MEK2 Amp F	CTCCCGGCCCGCCCCCTATG
MEK2 Amp R	GTGGAGGCGCCAGCCTGTCC
MEK2 Seq F	GTCAGCATCGCGGTTCTCC
MEK2 Seq R	TCACCCCGAAGTCACACAG
NRAS F	AGCTTGAGGTTCTTGCTGGT
NRAS R	TCAGGACCAGGGTGTCAGTG
AKT1 F	AGCGCCAGCCTGAGAGGA
AKT1 Amp R	TCTCCATCCCTCCAAGCTAT
AKT1 Seq R	GACAGGTGGAAGAACAGCT



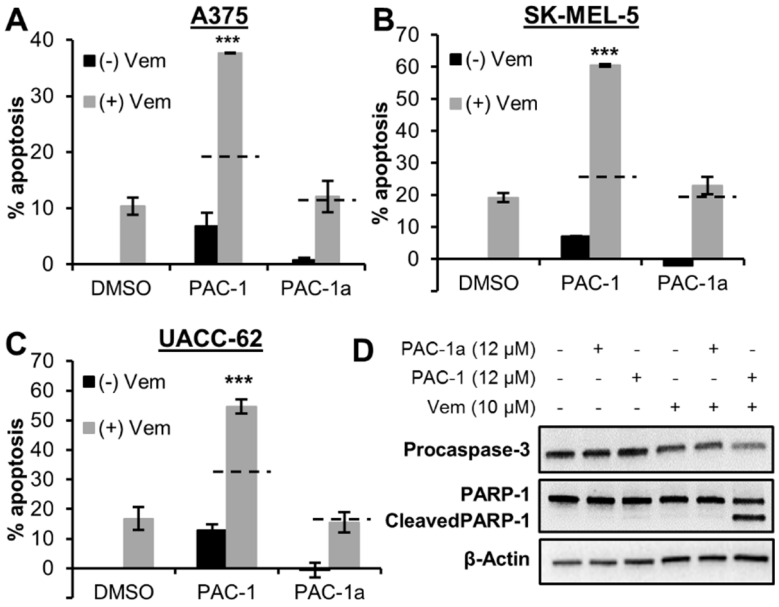
Supplementary Figure 1. Cell lines with ^{V600E}BRAF have significantly higher percent of cells undergoing apoptosis (assessed by Annexin V-FITC/PI staining) after treatment with vemurafenib (0.5 μM) and PAC-1 (12 μM) for 24 h, whereas this combination has negligible effect on cell lines with wild-type BRAF. Dashed horizontal lines represent the level of cell death expected from a mere additive effect of the two agents. Values are reported as mean ± SEM of at least three independent experiments. P-values shown for 2-way interaction to determine if the combination for induction of apoptosis is different from an additive effect (dashed horizontal lines) of individual agents are statistically significant (*** $p<0.001$).



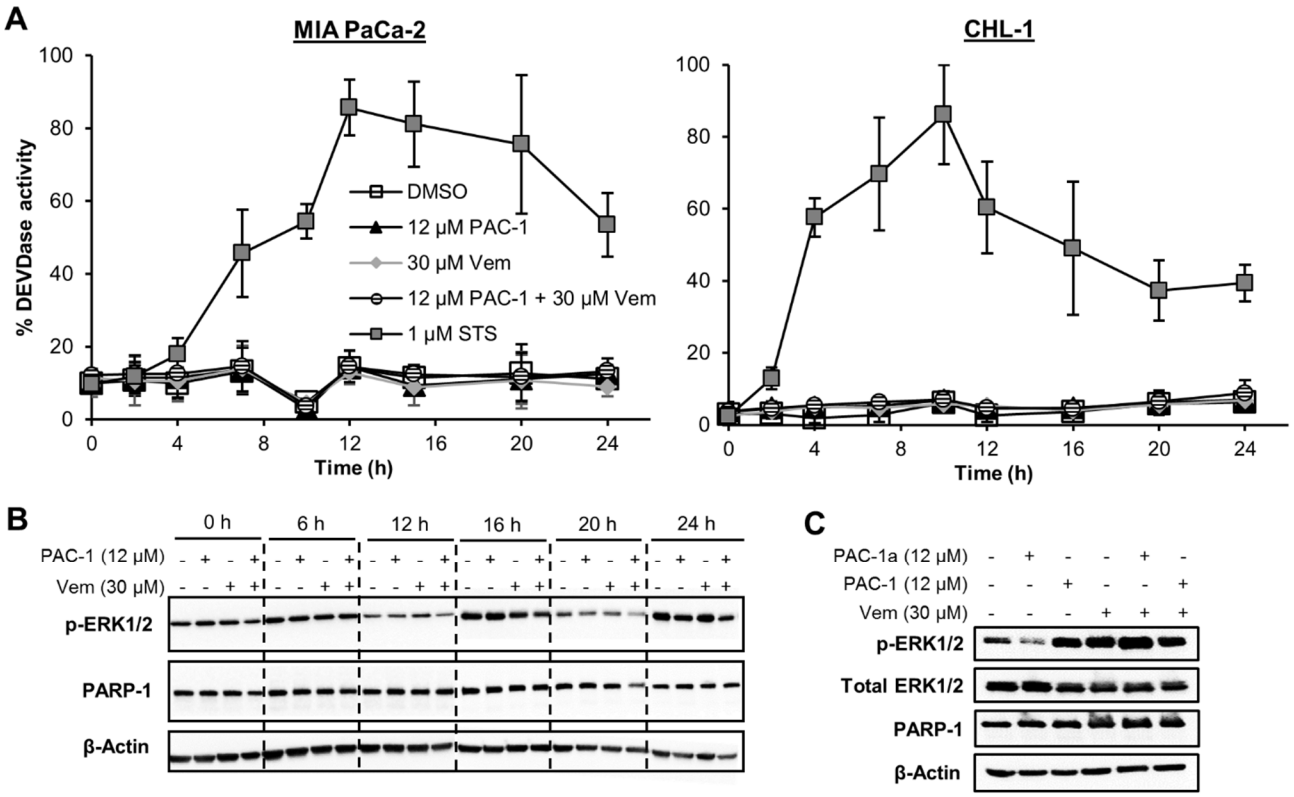
Supplementary Figure 2. PAC-1 and vemurafenib powerfully synergize to induce apoptotic death and caspase activity in SK-MEL-5 cells. (**A**) Shown is percent apoptotic cell death (assessed by Annexin V/PI staining and flow cytometry) induced after 24 h of treatment. Values shown are heat mapped with white representing low % apoptotic cell death and dark gray representing high % apoptotic cell death. (**B**) Combination indices (CI) calculated for each combination with Combosyn software. CI values are heat mapped with lowest values in light gray and the highest values in black. (**C**) Significant caspase-3/-7 enzymatic activity is observed in cells treated with the combination of PAC-1 and vemurafenib; PAC-1 (12 μM) and vemurafenib (10 μM) alone have little effect (p-values vs. DMSO control > 0.1 at all timepoints). Caspase-3/-7 activity in cell lysates was assessed with the fluorogenic Ac-DEVD-AFC substrate. Activity is expressed as normalized to minimal and maximal activity observed within the assay, with 1 μM staurosporine (STS) as the positive control. (**D**) PAC-1 (12 μM) and vemurafenib (10 μM) alone have little effect on PARP-1 cleavage in SK-MEL-5 cells, but significant PARP-1 cleavage is observed via western blot with the combination. (**E**) After 24 h, vemurafenib (0.5 μM and 1 μM) inhibited the phosphorylation of ERK1/2 with or without addition of PAC-1, indicating that effect of PAC-1 is downstream of the MAPK pathway. However, cleaved PARP-1 was only observed in cells treated with the vemurafenib/PAC-1 combination. Values are reported as mean ± SEM of at least three independent experiments. P-values shown for 2-way interaction to determine if the combination is different from additive are statistically significant at indicated timepoints. (* $p<0.05$, *** $p<0.001$)



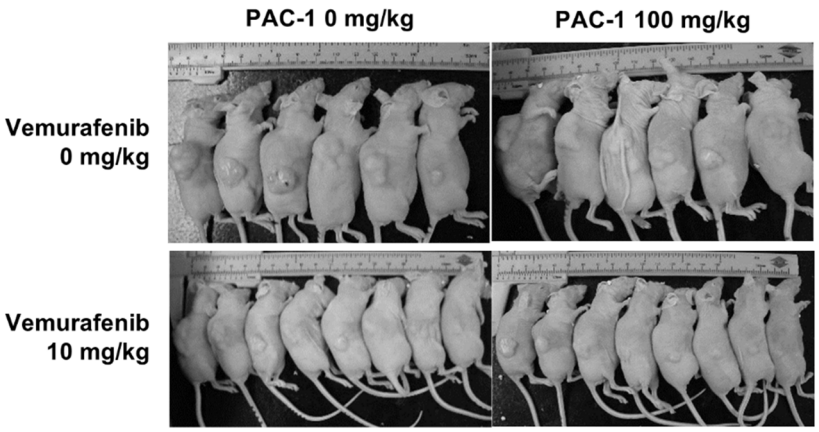
Supplementary Figure 3. PAC-1 and vemurafenib powerfully synergize to induce apoptotic death and caspase activity in UACC-62 cells. (**A**) Shown is percent apoptotic cell death (assessed by Annexin V/PI staining and flow cytometry) induced after 24 h of treatment. Values shown are heat mapped with white representing low % apoptotic cell death and dark gray representing high % apoptotic cell death. (**B**) Combination indices (CI) calculated for each combination with Combosyn software. CI values are heat mapped with lowest values in light gray and the highest values in black. (**C**) Significant caspase-3/-7 enzymatic activity is observed in cells treated with the combination of PAC-1 and vemurafenib PAC-1 (12 μM) and vemurafenib (10 μM) alone have little effect (p-values vs. DMSO control > 0.1 at all timepoints). Caspase-3/-7 activity in cell lysates was assessed with the fluorogenic Ac-DEVD-AFC substrate. Activity is expressed as normalized to minimal and maximal activity observed within the assay, with 1 μM STS as the positive control. (**D**) PAC-1 (12 μM) and vemurafenib (10 μM) alone have little effect on PARP-1 cleavage in UACC-62 cells, but significant PARP-1 cleavage is observed via western blot with the combination. (**E**) After 24 h, vemurafenib (0.5 μM and 1 μM) inhibited the phosphorylation of ERK1/2 with or without addition of PAC-1, indicating that effect of PAC-1 is downstream of the MAPK pathway. Minimal cleaved PARP-1 was observed in PAC-1 only treated cells, which was markedly increased in cells treated with the vemurafenib/PAC-1 combination. Values are reported as mean ± SEM of at least three independent experiments. P-values shown for 2-way interaction to determine if the combination is different from additive are statistically significant at indicated timepoints. (*** $p<0.001$)



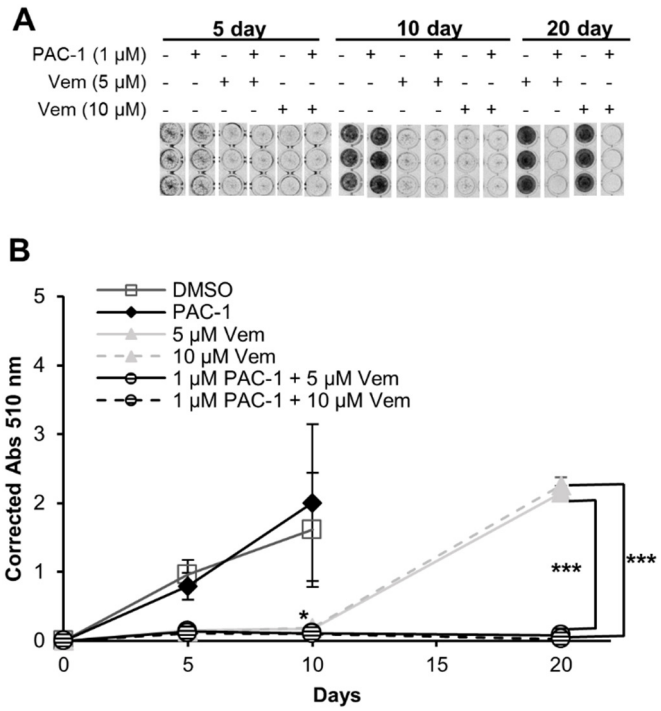
Supplementary Figure 4. Effect of PAC-1a (12 µM) vs PAC-1 (12 µM) in combination with vemurafenib (30 µM) in cell lines after 24 h treatment in (**A**) A375, (**B**) SK-MEL-5 and (**C**) UACC-62 cell lines as assessed by Annexin V-FITC/PI plots. Percent apoptosis reported is normalized relative to DMSO control sample. Dashed horizontal lines represent the level of cell death expected from a mere additive effect of the two agents. (**D**) PAC-1 (12 µM) and vemurafenib (10 µM) alone have minimal effect on PARP-1 cleavage in A375 cells, but increased PARP-1 cleavage is observed with the combination. PAC-1a (12 µM) in combination with vemurafenib (10 µM) does not increase PARP-1 cleavage. Values are reported as mean ± SEM of at least three independent experiments. P-values shown for 2-way interaction to determine if the combination for induction of apoptosis is different from an additive effect (dashed horizontal lines) of individual agents are statistically significant (*** $p<0.001$).



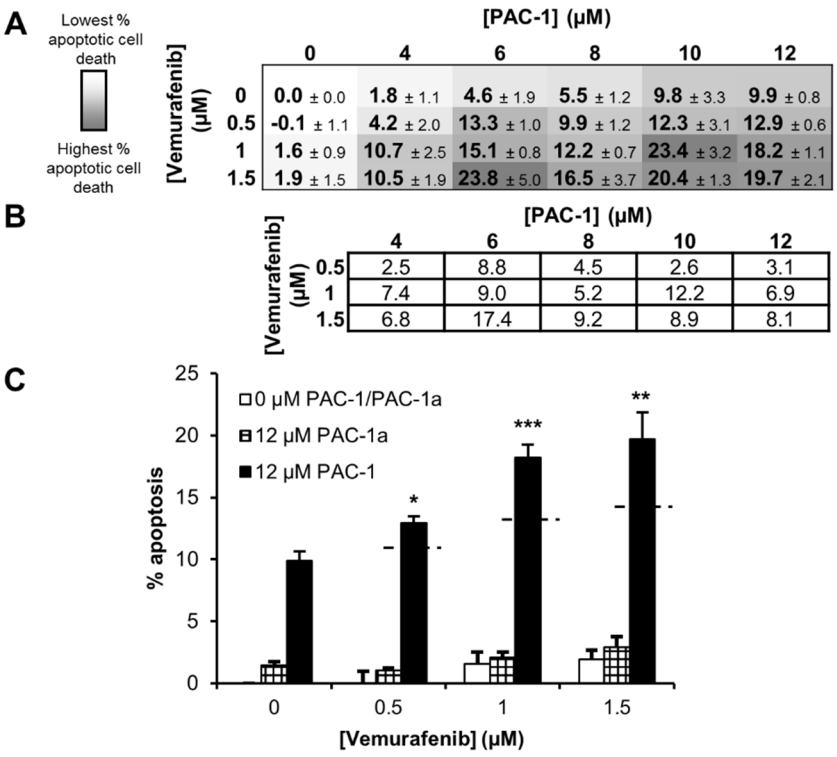
Supplementary Figure 5. Effect of the PAC-1 and vemurafenib combination in MIA PaCa-2 (mutant KRAS and ^{WT}BRAF) and CHL-1 (^{WT}KRAS and ^{WT}BRAF) cell lines with ^{WT}BRAF. (**A**) No effect on procaspase-3 activation is observed in MIA PaCa-2 and CHL-1 cell lines when treated with PAC-1 (12 μM) + vemurafenib (30 μM). Caspase-3/-7 activity in cell lysates was assessed with the fluorogenic Ac-DEVD-AFC substrate. Activity is expressed as normalized to minimal and maximal activity observed within the assay, with 1 μM STS as the positive control. (**B**) No effect on PARP-1 cleavage was observed in MIA PaCa-2 cells after 24 h. (**c**) PAC-1 (12 μM) and vemurafenib (30 μM) have no effect on PARP-1 cleavage in CHL-1 cells after 24 h treatment. Values are reported as mean ± SEM of at least three independent experiments.



Supplementary Figure 6. Images of tumor-bearing mice that were sacrificed after 15 days of continuous dosing. The four treatment groups are: control (n=6, 0 mg/kg PAC-1 and vemurafenib); mice treated once-a-day with 100 mg/kg PAC-1 (n=6), twice-a-day with 10 mg/kg vemurafenib (n=8), and the combination of 100 mg/kg PAC-1 (once-a-day) and 10 mg/kg vemurafenib (twice-a-day) (n=8).



Supplementary Figure 7. Addition of PAC-1 (1 μM) in the long-term treatment of UACC-62 cells with vemurafenib significantly delays cell regrowth. (**A**) UACC-62 cells were treated with PAC-1 (1 μM), vemurafenib (5 μM or 10 μM), or the combination. Media was washed out every 2-3 days and new compounds were added into each well. After 5, 10 or 20 days, the wells were fixed with 10% trichloroacetic acid, stained with 0.5% sulforhodamine B (SRB) dye, and imaged with BioRad GelDoc RX. Day 20 images of control and PAC-1 samples are not shown because the cells were unviable due to overcrowding. (**B**) Quantification of **(A)** where the SRB dye is dissolved in 10 mM Tris base at pH 10.4, and the absorbance read at 510 nm. Corrected absorbance at 510 nm was plotted against the days of continuous treatment by normalizing against absorbance on Day 0 before the start of treatment. Values are reported as mean ± SEM of at least three independent experiments. 2-tailed t-test performed between wells treated with vemurafenib only versus vemurafenib and PAC-1 (1 μM). On day 10, only the wells treated with vemurafenib (10 μM) and PAC-1 (1 μM) is significantly different from vemurafenib (10 μM) only ($p=0.035$) treatment. On day 20, wells treated with vemurafenib (5 or 10 μM) and PAC-1 (1 μM) are significantly different from vemurafenib (5 or 10 μM), as indicated on the graph. (* $p<0.05$, *** $p<0.001$)



Supplementary Figure 8. Effect of the PAC-1 and vemurafenib combination in A375VR cells. (**A**) Shown is percent apoptotic cell death (assessed by Annexin V/PI staining and flow cytometry) induced after 24 h of treatment. (**B**) The apoptotic cell death observed in (**A**) is greater than that predicted by the Bliss independent model. The excess cell death is calculated as $[f_{(observed,\ apoptotic)} - (f_{(PAC\text{-}1,\ apoptotic)} + f_{(vemurafenib,\ apoptotic)} - f_{(PAC\text{-}1,\ apoptotic)} * f_{(vemurafenib,\ apoptotic)})]*100\%$. This indicates that the observed effect is synergistic rather than additive. (**C**) The synergistic effect of PAC-1 and vemurafenib in activating apoptosis in A375VR after 24 h. This effect is abolished when the inactive PAC-1a was used. Dashed horizontal lines represent the level of cell death expected from a mere additive effect of the two agents. Values are reported as mean ± SEM of at least three independent experiments. P-values shown for 2-way interaction to determine if the combination for induction of apoptosis is different from an additive effect (dashed horizontal lines) of individual agents are statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Supplementary Table 1. Hematologic and biochemical toxicity of PAC-1 and vemurafenib. Average data from 4 mice treated with 100 mg/kg PAC-1 once-a-day and 10 mg/kg vemurafenib twice-a-day for 15 days. No clinically significant evidence for myelosuppression, renal injury, or hepatic toxicity was identified. *Platelet cell counts were low because platelet clumps were observed. Normal range values were obtained from Charles River for female NU/NU mice between 8 to 10 weeks of age.

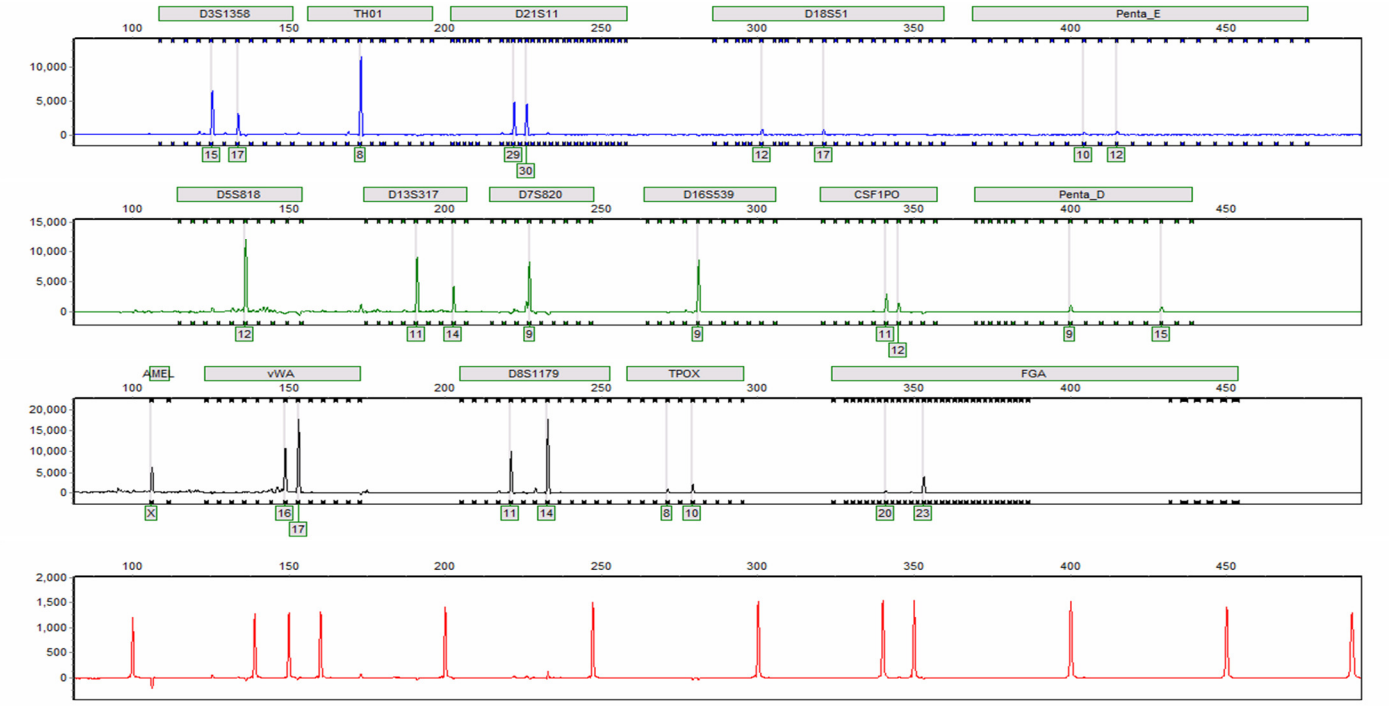
Blood chemistry	Ave ± SEM	Normal Range[1]
Creatinine (mg/dL)	0.20 ± 0.04	0.2 - 0.4
BUN (Urea) (mg/dL)	32.3 ± 1.0	11 - 39
Total Protein (g/dL)	4.7 ± 0.1	4.8 - 6.6
Albumin (g/dL)	2.2 ± 0.1	2.8 - 4.0
Globulin (g/dL)	2.5 ± 0.1	
Calcium (mg/dL)	9.2 ± 0.2	9.5 - 12.1
Phosphorous (mg/dL)	10.8 ± 0.5	8.0 - 15.5
Sodium (mmol/L)	161.0 ± 0.8	140.7 - 165.1
Potassium (mmol/L)	7.9 ± 0.2	7.0 - 10.8
Chloride (mmol/L)	119.0 ± 0.9	108.8 - 133.2
Glucose (mg/dL)	182.3 ± 12.5	149 - 271
Alkaline Phos Total (U/L)	70.8 ± 9.0	76 - 301
ALT (SGPT) (U/L)	50.5 ± 4.6	31 - 115
Total Bilirubin (mg/dL)	0.3 ± 0.1	0.2 - 0.5
Cholesterol total (mg/dL)	111.5 ± 4.4	98 - 202
Platelet Estimate* (10^3/µL)	229.3 ± 7.5	376 - 1796
WBC Estimate (10^3/µL)	3.2 ± 0.4	1.4 - 10.3
Seg %	31.5 ± 8.7	14.0 - 54.7
Lymph %	61.5 ± 9.6	23.6 - 79.3

Reference

1. Charles River Laboratories, Control data on NU/NU mice. (2012). Biochemistry and Hematology Data for NU/NU Mouse Colonies in North America for January 2008 – December 2011. Retrieved from http://www.criver.com/files/pdfs/rms/nunu/rm_rm_r_nunu_mouse_clinical_pathology_data.aspx

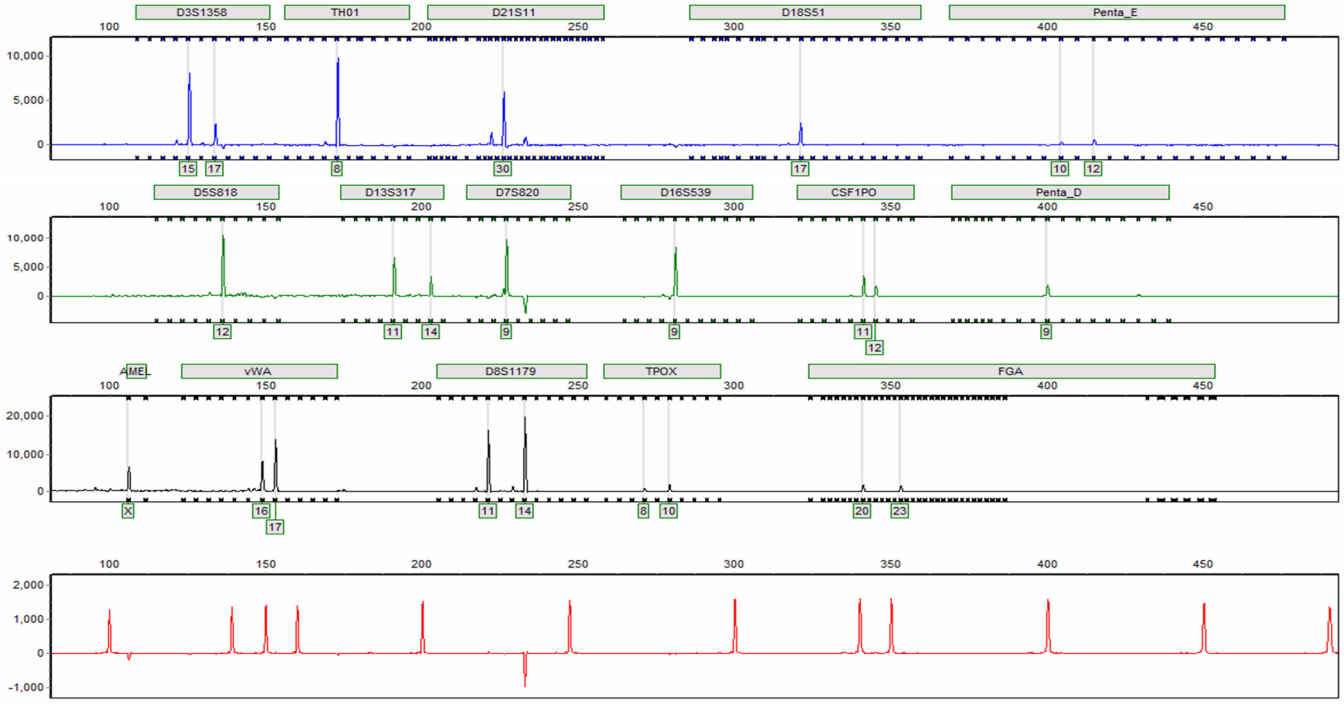
Appendix 1: Results from cell line authentication (Pages 12 – 19)

Sample 1: A375



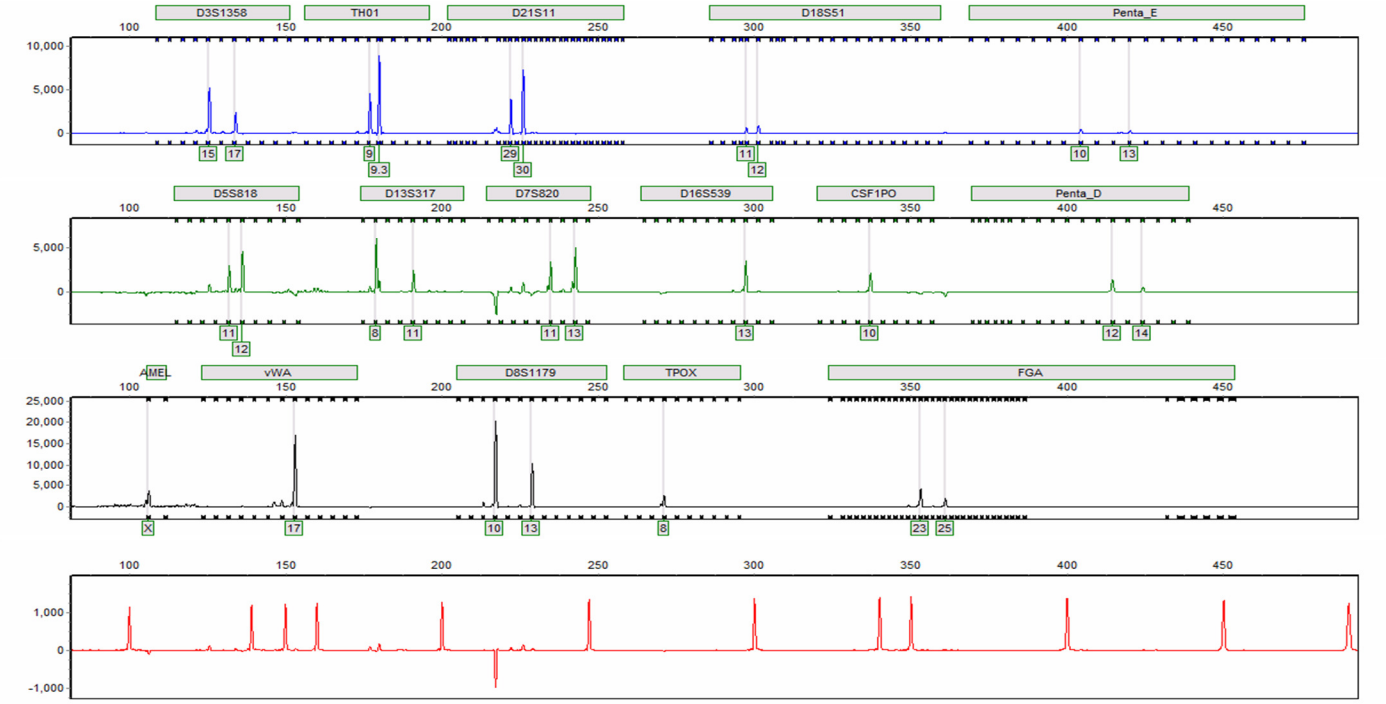
	D3S1358	TH01	D21S11	D18	Penta_E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta_D	AMEL	vWA	D8S1	TPOX	FGA	Notes
A375 Expected	15	8	29	12	10	12	11	9	9	11	9	X	16	11	8	20	
A375 Observed	17	8	30	17	12	12	14	9	9	12	15	X	17	14	10	23	Sample matches reference A-375 for all listed loci (DSMZ)

Sample 2: A375SM



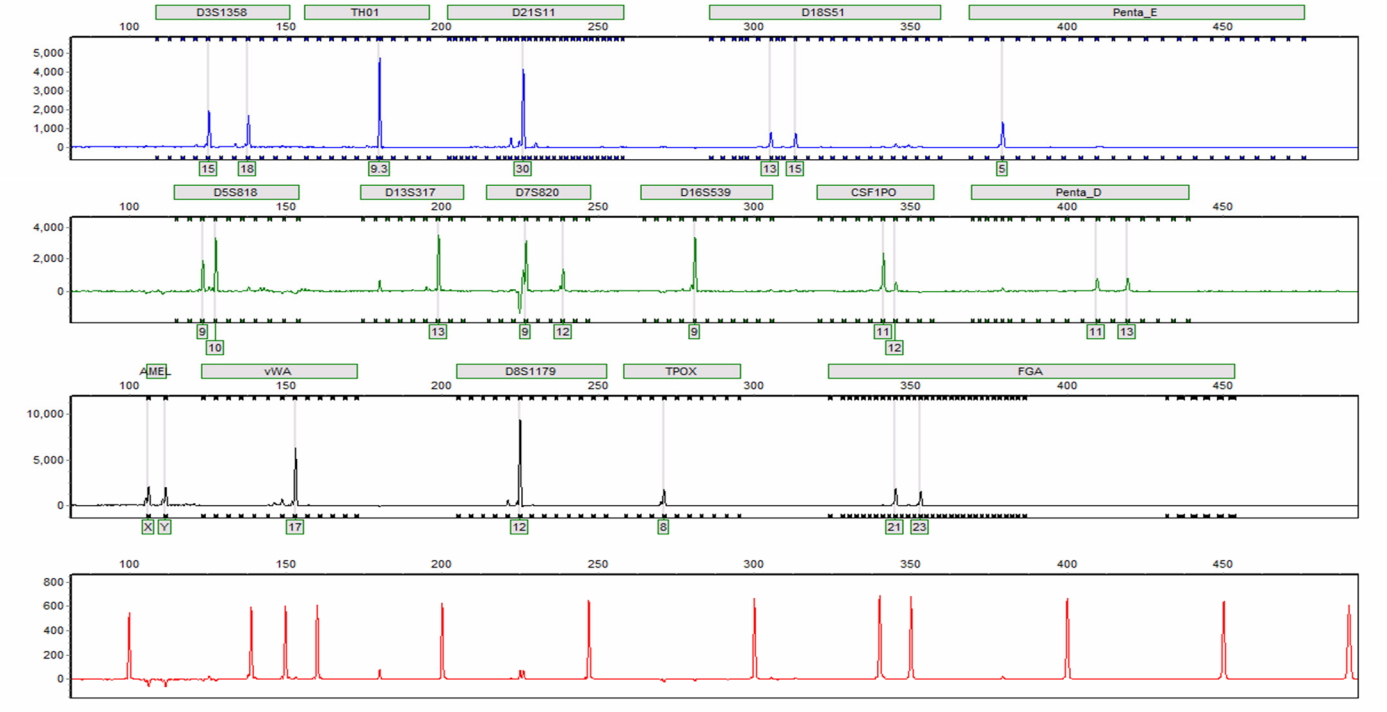
	D3S1358	TH01	D21S11	D18S51	Penta_E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta_D	AMEL	vWA	D8S1179	TPOX	FGA	Notes
A375SM Expected	15	8	30	17	10	12	11	9	9	11	9	X	16	11	8	20	
A375SM Observed	17	8	30	17	12	12	14	9	9	12	9	X	17	14	10	23	Sample matches reference A-375SM for all listed loci (DSMZ)

Sample 3: CHL-1



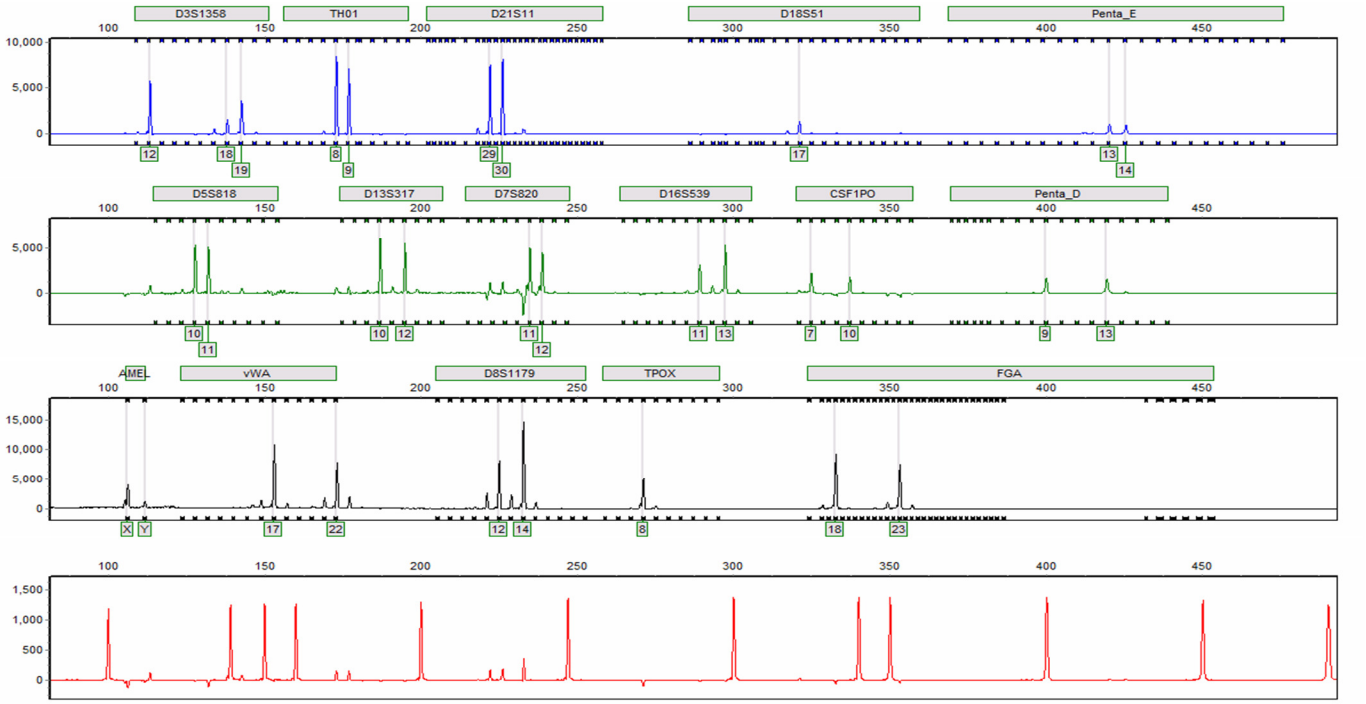
	D3S1358	TH01	D21S11	D18S51	Penta_E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta_D	AMEL	vWA	D8S1179	TPOX	FGA	Notes
CHL-1 Expected	15	9	29	11	10	11	8	11	13	10	12	X	17	10	8	23	
CHL-1 Observed	17	9.3	30	12	13	12	11	13	13	10	14	X	17	13	8	25	Sample matches reference CHL-1 for all listed loci (DSMZ)

Sample 4: H460



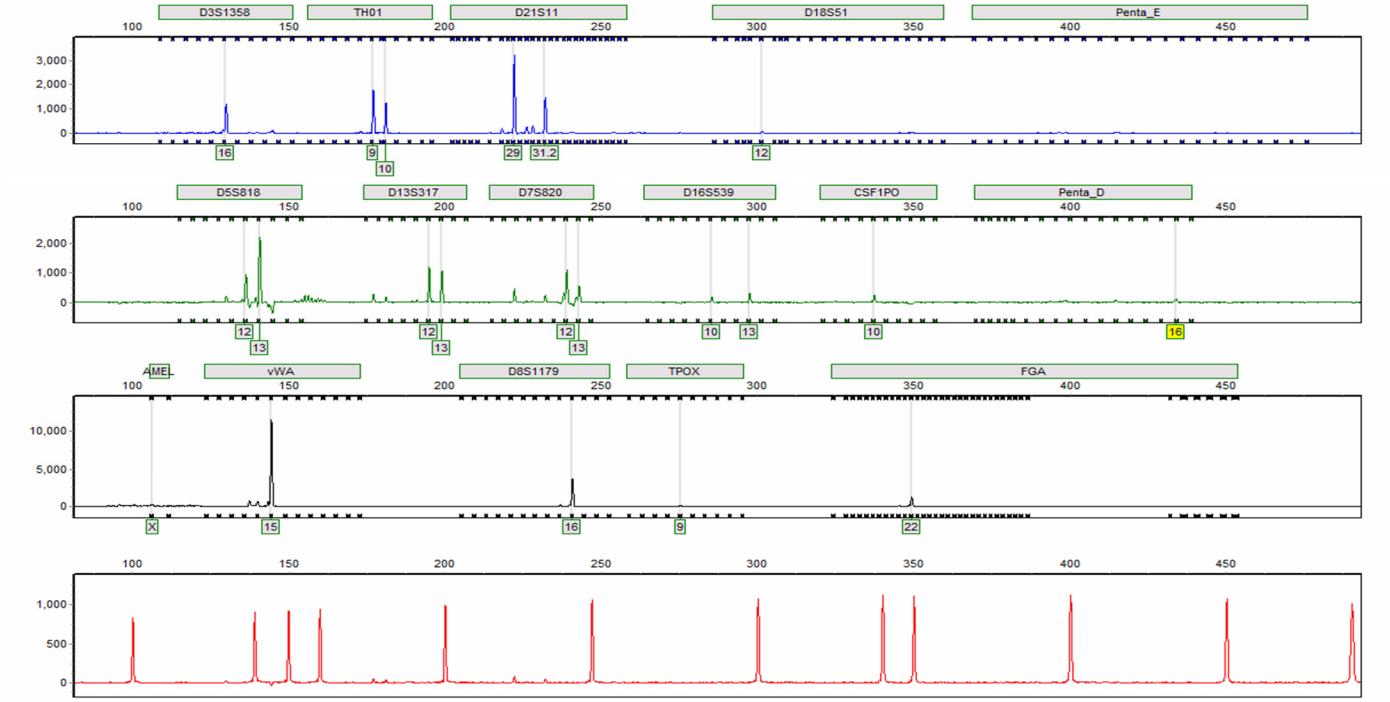
	D3S1358	TH01	D21S11	D18S51	Penta_E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta_D	AMEL	vWA	D8S1179	TPOX	FGA	Notes
H460 Expected	15	9.3	30	13	5	9	13	9	9	11	11	X	17	12	8	21	
H460 Observed	18	9.3	30	15	5	10	13	12	9	12	13	Y	17	12	8	23	Sample matches reference NCI-H460 for all listed loci (DSMZ)

Sample 5: HCT116



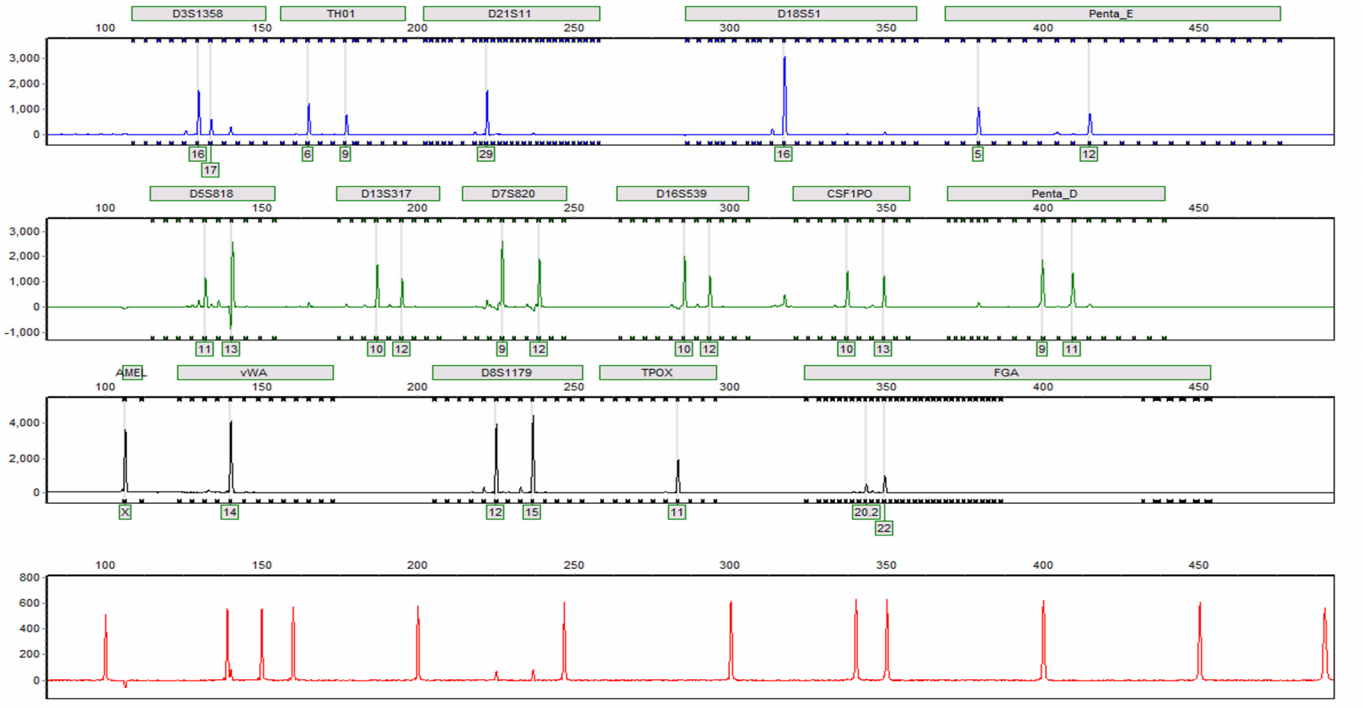
	D3S1358	TH01	D21S11	D18S51	Penta_E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta_D	AMEL	vWA	D8S1179	TPOX	FGA	Notes
HCT116 Expected	12	8	29	17	13	10	10	11	11	7	9	X	17	12	8	18	
HCT116 Observed	18+19	9	30	17	14	11	12	12	13	10	13	Y	22	14	8	23	Sample matches reference HCT 116 for all listed loci (DSMZ)

Sample 6: MIAPaCa2



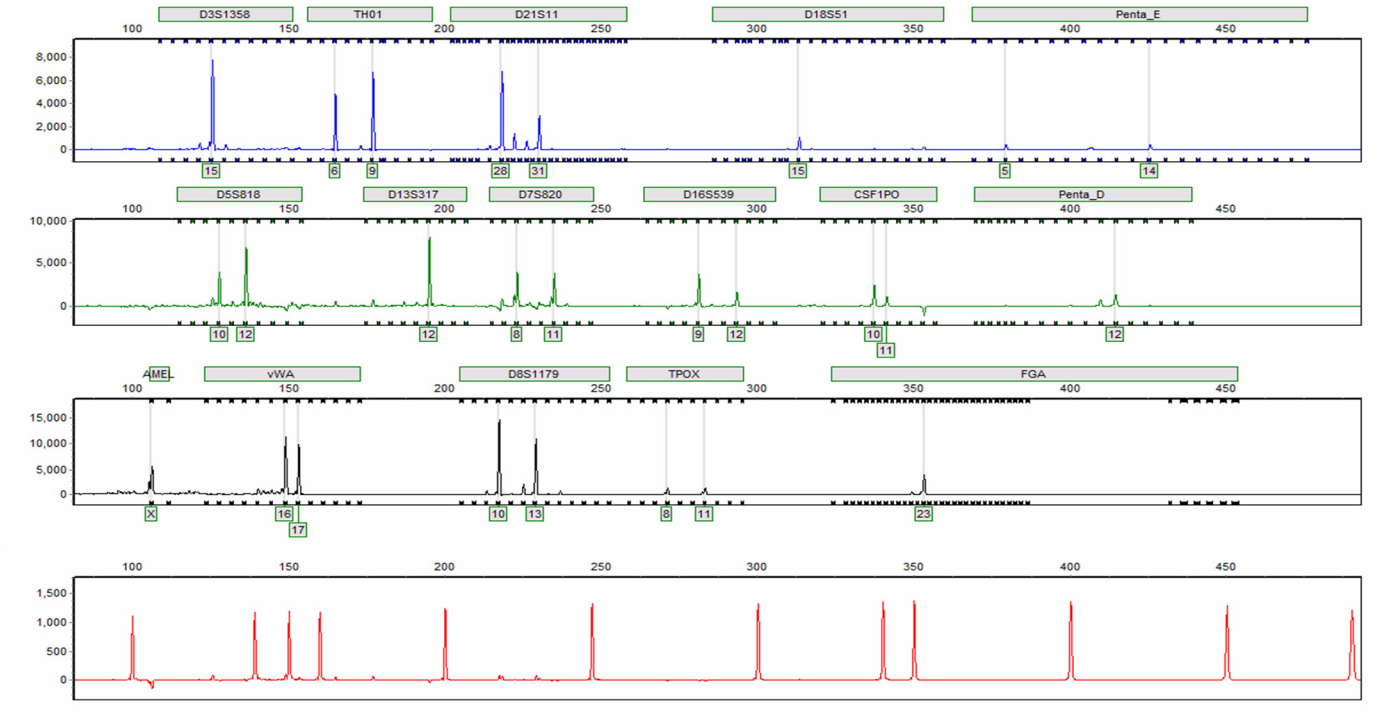
	D3S1358	TH01	D21S11	D18S51	Penta_E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta_D	AMEL	vWA	D8S1179	TPOX	FGA	Notes
MIAPaCa2 Expected	16	9	29	12		12	12	12	10	10	16	X	15	16	9	22	
MIAPaCa2 Observed	16	10	31.2	12		13	13	13	13	10	16	X	15	16	9	22	Sample matches reference MIA PaCa-2 for all listed loci (DSMZ). Penta E is not one of the reported loci.

Sample 7: SK-MEL-5



	D3S1358	TH01	D21S11	D18S51	Penta_E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta_D	AMEL	vWA	D8S1179	TPOX	FGA	Notes
SK-MEL-5 Expected	16	6	29	16	5	11	10	9	10	10	9	X	14	12	11	20.2	
SK-MEL-5 Observed	17	9	29	16	12	13	12	12	12	13	11	X	14	15	11	22	Sample matches reference SK-MEL-5 for all listed loci (DSMZ) except for a loss of heterozygocity at vWA (14,18 to 14,14).

Sample 8: UACC-62



	D3S1358	TH01	D21S11	D18S51	Penta_E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta_D	AMEL	vWA	D8S1179	TPOX	FGA	Notes
UACC-62 Expected	15	6	28	15	5	10	12	8	9	10	12	X	16	10	8	23	
UACC-62 Observed	15	9	31	15	14	12	12	11	12	11	12	X	17	13	11	23	Matches reference profile UACC-62 at all listed loci on The Wistar Institute website.

Appendix 2: Results of mycoplasma test (A375 cell line)

ILLINOIS

VETERINARY DIAGNOSTIC LABORATORY
COLLEGE OF VETERINARY MEDICINE
2001 S Lincoln Ave
Urbana, IL 61802
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T: (217) 333-1620 F: (217) 244-2439

Final Report

U of I Teaching Hospital
Vet: TIMOTHY FAN
1008 W. HAZELWOOD DR
URBANA, IL 61802

Case#: 15-34418
Accessioned: 05/13/15
Report Generated: 05/14/15 @ 4:00 PM
Results Last Modified: 05/14/15 @ 3:26PM

Case ID	Owner	Coordinator
Peh	Peh, Jessie	MADDOX
Breed	**Species**	**Sex / Neutered Age**
Unknown Species (Reported As)	Unknown Species (Reported As)	N / R

Microbiology

Mycoplasma detect PCR - Verified: 05/14/15 3:26 PM by NJ1

Specimen	Specimen ID	Results
Culture material	A375-Human Cell Line	Negative