

## Supplemental Data

## Structure of the Pseudokinase VRK3 Reveals a Degraded Catalytic Site, a Highly Conserved Kinase Fold, and a Putative Regulatory Binding Site

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**Table S1. Crystallographic Data and Refinement Statistics**

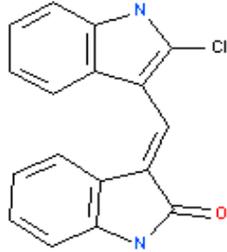
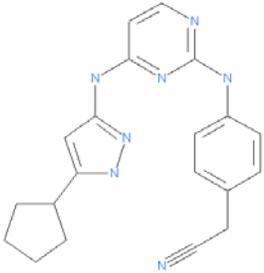
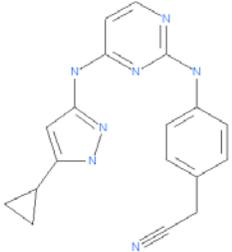
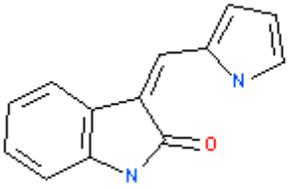
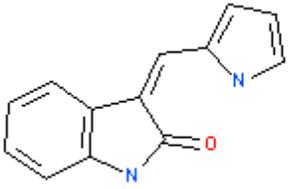
Data Collection	VRK2	VRK3
Space group	P2 <sub>1</sub> 2 <sub>1</sub> 2	P2 <sub>1</sub>
Cell dimensions [Å and °]	a=70.65, b=157.53, c=56.29	a=91.26, b=54.42, c=91.82, β=91.2
Resolution*	1.7 (1.7-1.8)	2.0 (2.0-2.1)
Unique observations*	67759 (10022)	60969 (8014)
Completeness* [%]	96.9 (93.3)	99.2 (95.4)
Redundancy*	5.8 (5.5)	3.6 (2.6)
<i>R</i> <sub>merge</sub> *	0.0562 (0.5859)	0.0648 (0.4167)
<i>I</i> / $\sigma I$ *	15.3 (2.3)	12.5 (2.2)
<b>Refinement</b>		
Reflections ( <i>R</i> <sub>free</sub> set)	64302 (3413)	57853 (3092)
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub>	0.201 / 0.236	0.178 / 0.223
Atoms (P/L/W) <sup>#</sup>	4828 / 21 / 334	5143 / 20 / 514
B-factors (P/L/W) <sup>#</sup> [Å <sup>2</sup> ]	34.1 / 50.0 / 38.5	34.9 / 49.0 / 40.8
RMSD bonds [Å]	0.015	0.011
RMSD [°]	1.483	1.265
<b>Ramachandran</b>		
favorable [%]	99.6	100.0
allowed [%]	0.4	0.0

**Table S2. Superpositions between VRK2 and VRK3 and Other Representative Protein Kinases, as Aligned by DaliLite (Holm and Park, 2000)**

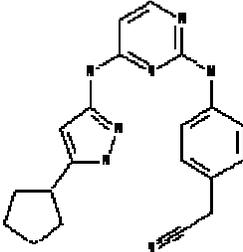
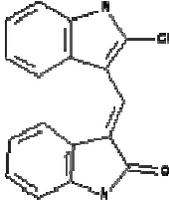
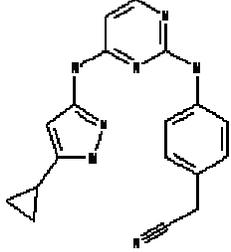
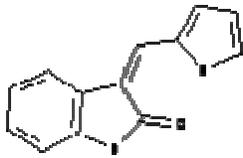
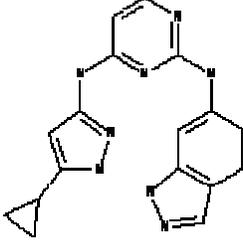
Structure (PDB ID: Chain)	VRK3 (2JII:B)	VRK2 (2V62:A)	CK1 (1CSN:A)	PKA (1CDK:A)
<b>VRK2</b> (2V62:A)	2.3 (291,38,35.4)			
<b>CK1</b> (1CSN:A)	2.1 (271,23,30.3)	2.4 (266,27,29.2)		
<b>PKA</b> (1CDK:A)	3.1 (257,16,22.2)	3.1 (241,18,20)	2.8 (258,19,23.9)	
<b>Sky1p</b> (1Q97:A)	3.0 (272,15,23.6)	3.1 (262,19,21)	2.6 (250,20,22.9)	2.7 (251,26,24)

RMSD is given in Å, followed by additional information on the structural comparison in parentheses: number of aligned positions, % sequence identity, and DaliLite Z-score. Comparisons between VRK3 and VRK2 and paralogues are highlighted in gray.

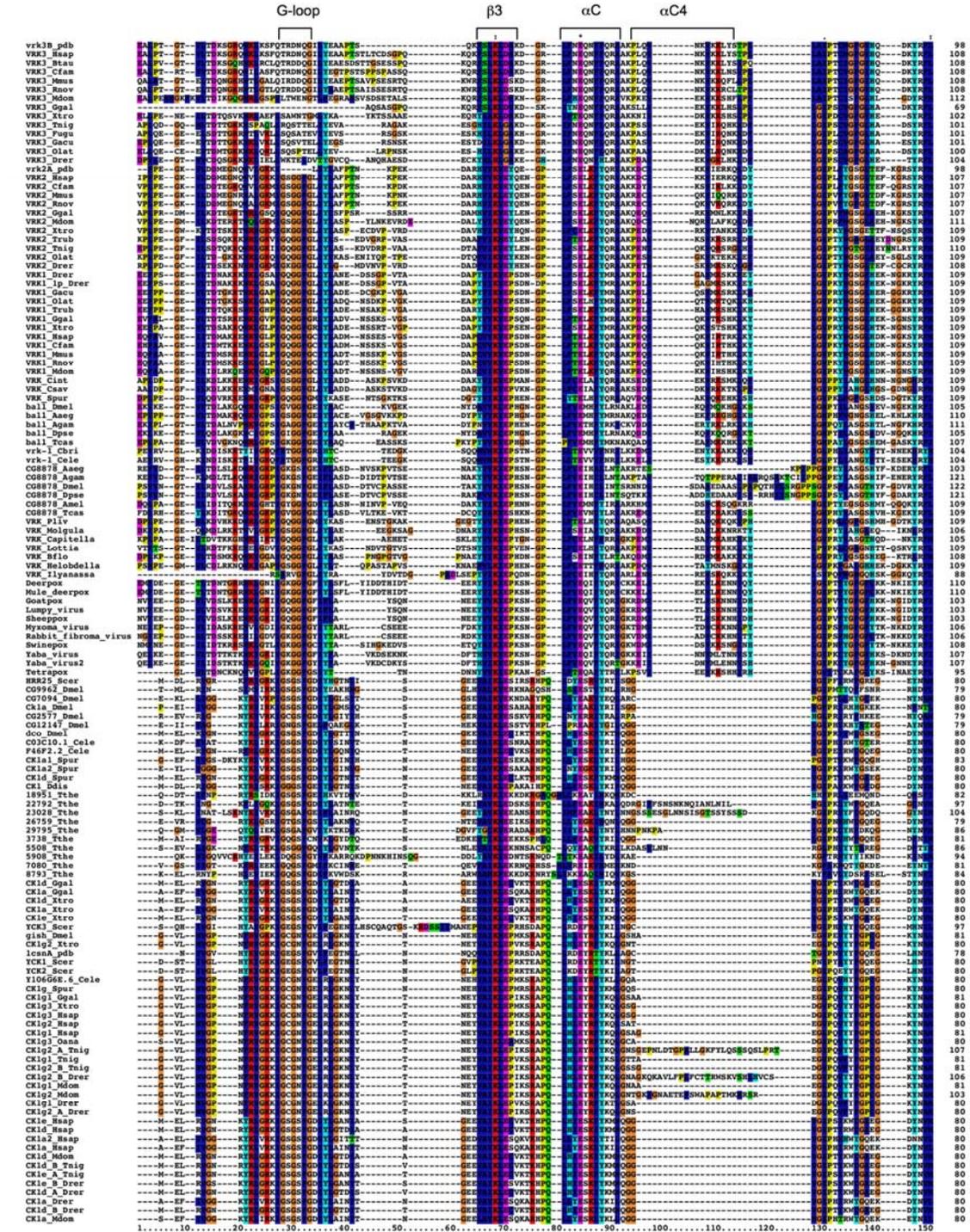
**Table S3. Inhibitor Screening Results for VRK1**

Compound name	Chemical structure	VRK1 T <sub>m</sub> Shift [°C]
3-(2-Chloro-3-indolylmethylene)-1,3-dihydroindol-2-one	 <p>The structure shows a 1,3-dihydroindol-2-one core with a carbonyl group at position 2 and a 2-chloro-3-indolylmethylene group at position 3. The chlorine atom is highlighted in black, and the nitrogen atoms are highlighted in blue.</p>	4
ASC67	 <p>The structure features a central pyrimidine ring connected via nitrogen atoms to a 1H-imidazole ring (with a cyclopentyl group at position 2) and a 4-(cyanomethyl)phenyl ring.</p>	4.3
ASC24	 <p>The structure is similar to ASC67, but the imidazole ring is substituted with a cyclopropyl group at position 2.</p>	4.5
Oxindole I; 3-(1H-Pyrrol-2-ylmethylene)-1,3-dihydroindol-2-one	 <p>The structure shows a 1,3-dihydroindol-2-one core with a carbonyl group at position 2 and a 1H-pyrrol-2-ylmethylene group at position 3. The nitrogen atoms are highlighted in blue.</p>	3.5
5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]-2-indolinone	 <p>The structure shows a 2-indolinone core with a carbonyl group at position 2 and a (3,5-dibromo-4-hydroxyphenyl)methylene group at position 3. The nitrogen atom is highlighted in blue.</p>	3.5

**Table S4. Inhibitor Screening Results for VRK2**

Compound name	Chemical structure	VRK2 T <sub>m</sub> Shift [°C]
ASC67		4.5
3-(2-Chloro-3-indolylmethylene)- 1,3-dihydroindol-2-one		4
ASC24		4
"Oxindole I; 3-(1H-Pyrrol-2-ylmethylene)-1,3-dihydroindol-2-one"		3.5
ASC29		3.5

**Figure S1. Curated Alignment of VRKs and CK1s**  
 Key sections discussed in text are highlighted.



αE Catalytic Loop β7

vrk38_pdb	PS	QMS	DS	SP	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	157			
VRK3_Hsasp	PT	GR	AS	DD	FR	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	167		
VRK3_Cfau	PI	GR	AS	DD	FR	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	167		
VRK3_Mmus	PI	GR	AS	DD	FR	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	167		
VRK3_Raov	PS	GR	AS	DD	FR	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	167		
VRK3_Mdom	PD	GR	AS	DD	FR	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	171		
VRK3_Ggal	SD	GR	AS	DD	FR	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	124		
VRK3_Xtro	SA	QQA	AS	DD	DD	DK	PE	EA	IE	EL	MT	IT	HE	ME	GG	IT	SI	D	161	
VRK3_Tnig	SD	GR	AS	EE	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	155		
VRK3_Fupu	SN	GR	AS	EE	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	159		
VRK3_Gacu	PH	GR	AS	EE	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	159		
VRK3_Olat	SH	GR	AS	EE	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	158		
VRK3_Drer	PC	GR	AS	EE	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	162		
vrk2a_pdb	SK	GI	DK	--	DD	GR	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	155
VRK2_Hsasp	SK	GI	DK	--	DD	GR	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	164
VRK2_Cfau	SK	GI	DK	--	DD	GR	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	164
VRK2_Mmus	SK	GI	DK	--	DD	GR	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	164
VRK2_Raov	SK	GI	DK	--	DD	GR	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	164
VRK2_Ggal	SK	GI	DK	--	DD	GR	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	164
VRK2_Mdom	SK	GI	DK	--	DD	GR	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	168
VRK2_Xtro	SK	GI	DK	--	DD	GR	KK	RV	SR	ES	Q	R	DA	HE	ME	GG	IT	SI	D	167
VRK2_Trub	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK2_Tnig	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	168	
VRK2_Olat	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK2_Drer	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	166	
VRK1_Drer	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_lp_Drer	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Gacu	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Olat	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Trub	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Ggal	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Xtro	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Hsasp	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Cfau	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Mmus	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Raov	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Mdom	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK1_Glat	SR	GD	DR	ERS	RS	R	FK	LA	Q	DR	SV	IT	HE	ME	GG	IT	SI	D	167	
VRK_Csasp	PR	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	167	
VRK_Spar	PL	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	167	
ball_Dmel	PR	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	163	
ball_Aaag	PR	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	168	
ball_Agam	PR	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	169	
ball_Dpse	PR	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	163	
ball_Tcas	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	164	
vrk-1_Cele	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	162	
vrk-1_Cele	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	162	
CG8878_Aaag	KK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	252	
CG8878_Agam	KK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	252	
CG8878_Dmel	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	253	
CG8878_Dpse	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	257	
CG8878_Amel	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	263	
CG8878_Tcas	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	263	
VRK_Pliv	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	167	
VRK_Molpala	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	165	
VRK_Capitella	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	163	
VRK_Lotia	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	167	
VRK_Hilo	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	124	
VRK_Melobdella	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	167	
VRK_Tlyanassa	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	146	
Deerpox	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	168	
Male_deerpox	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	168	
Goatpox	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	161	
Lumpy_virus	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	161	
Sheepox	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	161	
Myxoma_virus	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	159	
Rabbit_fibroma_virus	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	164	
Swinepox	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	166	
Yaba_virus	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	164	
Yaba_virus2	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	165	
Tetrapox	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	153	
RR25_Scar	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
CG9962_Dmel	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	137	
CG7094_Dmel	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
Ck1a_Dmel	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
CG2577_Dmel	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	137	
CG1247_Dmel	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
dco_Dmel	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
CG1010_Cele	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
F48F7.1_Cele	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
Ck1a1_Spur	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	141	
Ck1a2_Spur	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
Ck1d_Spur	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
Ck1_Edis	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	138	
18951_Ttbe	PK	GD	DR	ER	L	K	L	K	L	K	L	K	L	K	L	K	L	K	139	
21792_Ttbe	PK	GD	DR	ER																

vrk3B_pdb	157
VRK3_Hsap	167
VRK3_Btau	167
VRK3_Cfam	167
VRK3_Mmus	167
VRK3_Rnov	167
VRK3_Mdom	171
VRK3_Ggal	126
VRK3_Xtro	161
VRK3_Tnig	159
VRK3_Fugu	159
VRK3_Gacu	159
VRK3_Olat	158
VRK1_Drer	162
vrk2A_pdb	155
VRK2_Hsap	164
VRK2_Cfam	164
VRK2_Mmus	164
VRK2_Rnov	164
VRK2_Ggal	164
VRK2_Mdom	168
VRK2_Xtro	166
VRK2_Trub	167
VRK2_Tnig	168
VRK2_Olat	167
VRK2_Drer	166
VRK1_Drer	167
VRK1_Ip_Drer	167
VRK1_Gacu	167
VRK1_Olat	167
VRK1_Trub	167
VRK1_Ggal	167
VRK1_Xtro	167
VRK1_Hsap	167
VRK1_Cfam	167
VRK1_Mmus	167
VRK1_Rnov	167
VRK1_Mdom	167
VRK_Cint	167
VRK_Csav	167
VRK_Spur	167
ball_Dmel	163
ball_Aaeg	168
ball_Agam	169
ball_Dpse	163
ball_Tcas	164
vrk-1_Cbri	162
vrk-1_Cele	162
CG878_Aaeg	326
CG878_Agam	344
CG878_Dmel	360
CG878_Dpse	415
CG878_Amel	167
CG878_Tcas	166
VRK_Pliv	167
VRK_Molpula	165
VRK_Capitella	163
VRK_Lottia	167
VRK_Bilo	126
VRK_Melobdella	167
VRK_Ilyanassa	146
Deerpox	168
Mula_deerpox	168
Goatpox	161
Lumpy_virus	161
Ebepox	161
Myxoma_virus	164
Rabbit_fibroma_virus	164
Swinepox	166
Yaba_virus	165
Yaba_virus2	165
Tatrapox	163
RRR25_Scer	138
CG9962_Dmel	138
CG7094_Dmel	138
Ck1a_Dmel	138
CG2577_Dmel	137
CG12147_Dmel	137
cco_Dmel	138
CG10101_Cele	138
F46F2.2_Cele	138
CK1a1_Spur	141
CK1a2_Spur	138
CK1d_Spur	138
CK1_Ddis	138
18951_Ttbe	139
22792_Ttbe	155
23028_Ttbe	162
26759_Ttbe	137
29795_Ttbe	144
3738_Ttbe	139
5508_Ttbe	144
5908_Ttbe	152
7080_Ttbe	139
8753_Ttbe	142
CK1d_Ggal	138
CK1a_Ggal	138
CK1d_Xtro	138
CK1a_Xtro	148
CK1a_Xtro	148
YCK3_Scer	155
gish_Dmel	139
CK1g_Xtro	138
lcanA_pdb	136
YCK1_Scer	138
YCK2_Scer	138
Y106668.6_Cele	138
CK1g_Spur	138
CK1g1_Ggal	139
CK1g1_Xtro	138
CK1g1_Hsap	138
CK1g2_Hsap	138
CK1g1_Hsap	139
CK1g3_Oma	138
CK1g2_A_Tnig	165
CK1g1_Tnig	139
CK1g2_B_Tnig	139
CK1g2_B_Drer	144
CK1g1_Mdom	139
CK1g2_Mdom	161
CK1g1_Drer	138
CK1g2_A_Drer	138
CK1e_Hsap	138
CK1d_Hsap	138
CK1a2_Hsap	138
CK1a_Hsap	148
CK1d_Mdom	138
CK1d_B_Tnig	138
CK1a_A_Tnig	138
CK1e_B_Drer	138
CK1d_A_Drer	138
CK1a_Drer	138
CK1d_B_Drer	138
CK1a_Mdom	138

.....310.....320.....330.....340.....350.....360.....370.....380.....390.....400.....410.....420.....430.....440.....450

