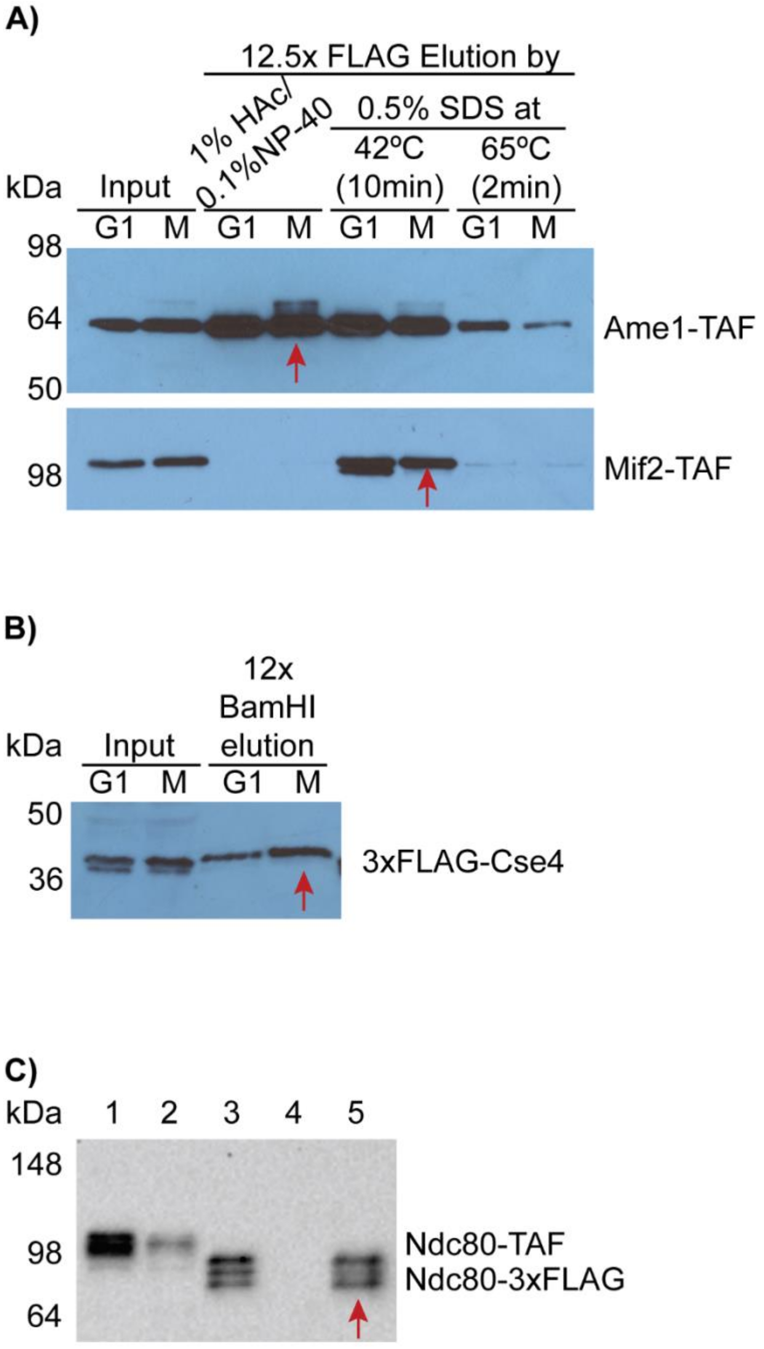
S1 Figure. Western blot analysis the immuno-precipitation of TAF-tagged kinetochore subunits. TAF tag: 3xFlag-tev-ProteinA. The purified samples were then processed and analyzed by data-dependent acquisition MS analysis.



Red arrows indicate the samples processed for DDA-MS.

A) M2 FLAG purification of Ame1-TAF and Mif2-TAF from α factor (G1) or Cdc20 auxin degradation (M) arrested cells. HAc stands for acetic acid. (Anti-protein A Western blots)

B) *Ex vivo* reconstitution of G1 or M phase arrested 3xFLAG-Cse4 strain. (Anti-FLAG Western blot)

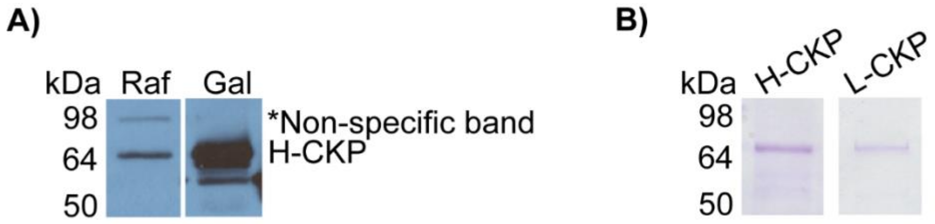
C) 2-step purification of Ndc80 from M phase arrested Ndc80-TAF strain. The first step was done using IgG beads, followed by TEV protease cleavage to cut at the TEV site between protein A and 3xFLAG. The second step was done using M2 FLAG beads, followed by elution with 1% acetic acid/0.1% NP-40. (Anti-FLAG Western blot)

1 Input
2 IgG flowthrough
3 6x TEV proteose elution
4 M2 FLAG flowthrough
5 15x FLAG elution

“x” indicate loading relative to input.

↑ indicate sample used for DDA-MS.

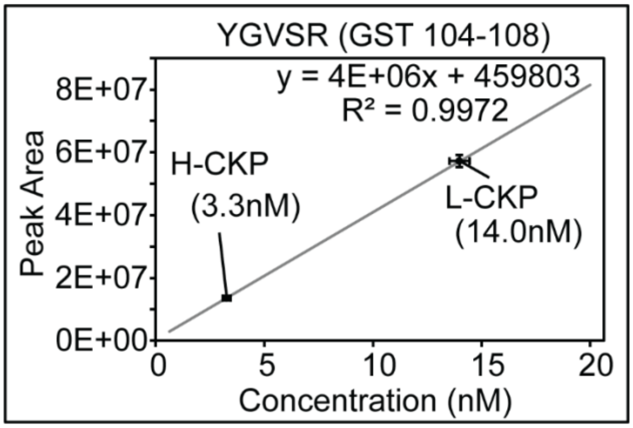
S2 Figure. Evaluate CKP expression in yeast by anti-Flag WB and purification via anti-Flag resins.



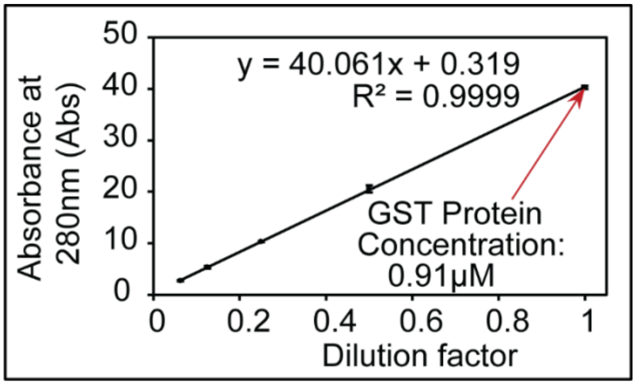
A) Anti-FLAG Western blot for galactose induction of H-CKP. Raf: raffinose, Gal: galactose.

B) Coomassie brilliant blue staining of H and L-CKP after purification. Staining for L and H-CKP was done on different days on different gels.

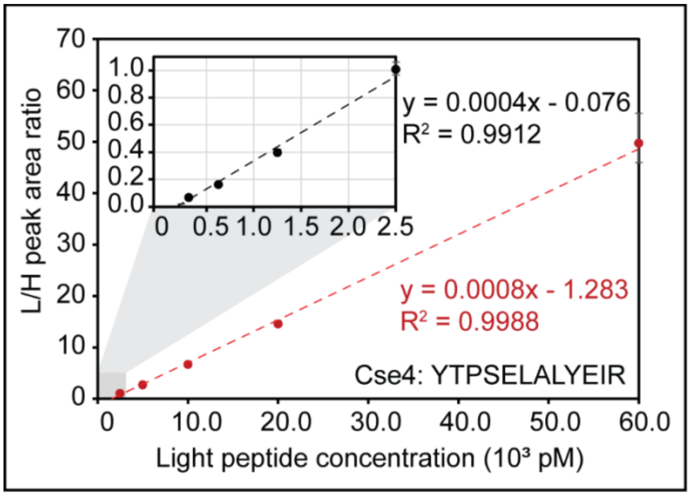
S3 Figure. Absolute quantification of L and H-CKP using a commercially synthesized GST peptide (sequence YGVSR) via external calibration.



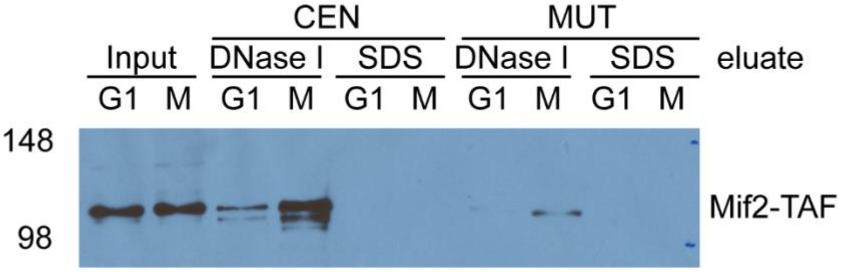
S4 Figure. The concentration of recombinant GST protein determined by UV-Vis absorption, which was then used to quantify H-CKP and L-CKP by MS.



S5 Figure. Cse4 has 2 calibration curves covering its full AMR, one at a lower concentration below 2.5 nM, one at the higher concentration up to 60 nM.

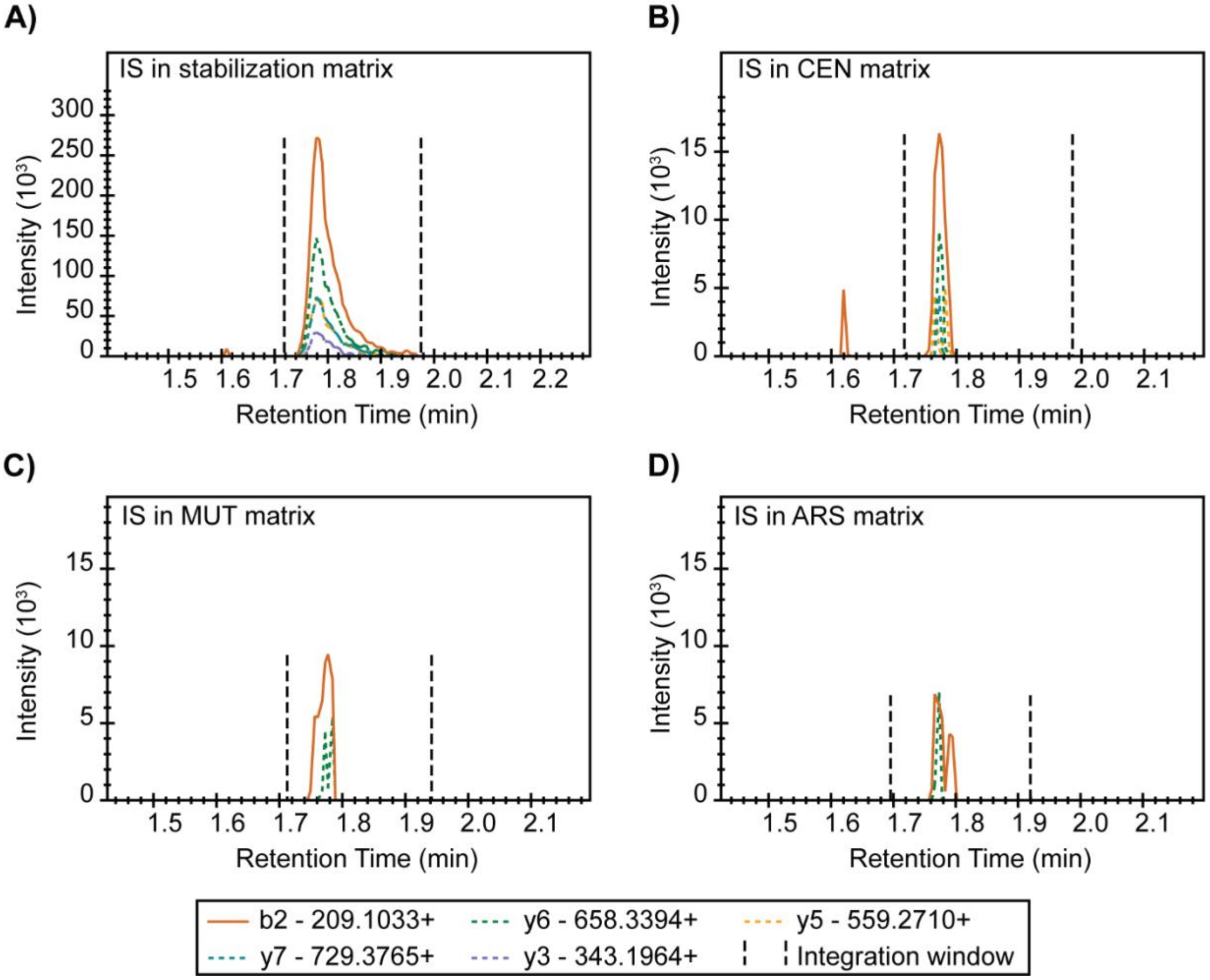


S6 Figure. Evaluation of *ex vivo* kinetochore reconstitution using Mif2-TAF cell extracts



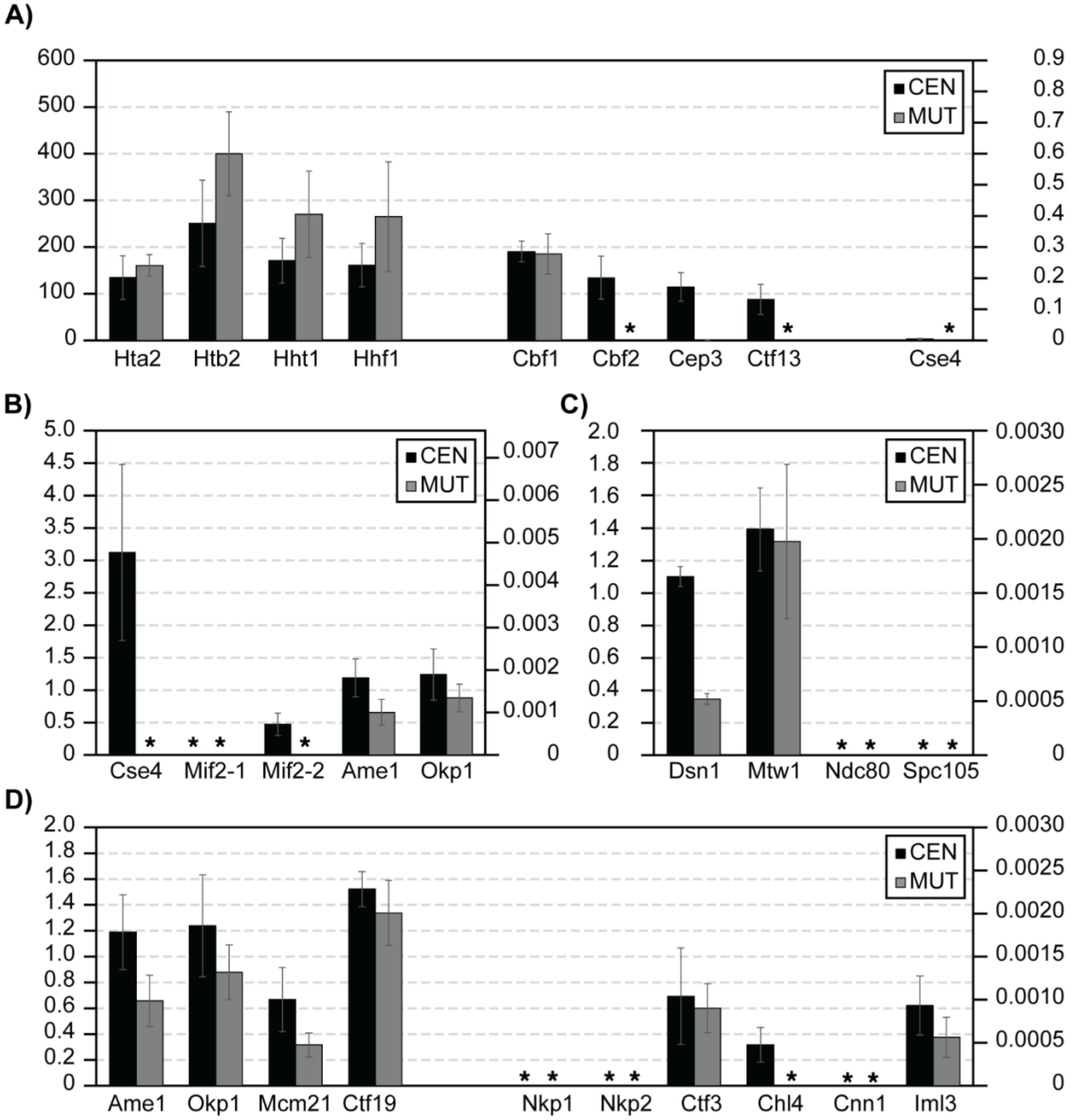
M-phase extract has a stronger Mif2-CEN association than G1 extract. Moreover, DNase-I elution is complete in eluting Mif2 since the subsequent SDS elution yielded much fewer signals. Mif2-CEN association is also specific and reduced by point mutation to the CDE III region in CEN3 (MUT).

S7 Figure. Htb2 peptide chromatograph shows sample-specific matrix suppression. IS: Internal standard, referring to Htb2 peptide here.



Chromatograms of indicated transition ions of the Htb2 peptide (HAVSEGTR) from the H-CKP. Y-axes indicate signal intensity, and x-axes indicate retention time in minutes. Black dotted lines indicate the integration windows for determining the peak area of each transition ion.

S8 Figure. ***Ex vivo*** **reconstitution of kinetochores using G1-phase lysate**



Primary y-axis (left): Normalized concentration (nM)
Secondary y-axis (right): Protein/DNA ratio
*Below limit of quantification

S1 Table. Plasmids and yeast strains.

Plasmid	**Description/source**
HZE2029	Lic-2GT from Corbett Lab
HZE3236	pRS423-pGAL-GST-tev-HF
HZE3240	Addgene 30116, pFastBacCEN3e2_Plus60_tetO2, from Hinshaw: pSMH1717
HZE3241	Addgene 30116, pFastBac LIC cloning vector, CEN3e2_Plus60-TetO2-CCGmut, from Hinshaw: pSMH1726
HZE3246	CEN3-8xLacO-TRP1, from Biggins: pSB964
HZE3361	pRS423-pGAL-GST-CKP-HF

Yeast Strain	**Genotype**
SCY249	*ura3-52 leu2-1 trp1-63 his3-200 lys2-Bgl hom3-10 ade2-1 ade8 sml1Δ::TRP arg4Δ MAT a*
HZY1029	*bar1Δ::URA MAT a*, W303 in HZY1077
HZY1077	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5+*, *MAT a*, W303
HZY2347	*Cdc20-HA-AID::G418 pGPD1-OsTir1::LEU2 bar1Δ::URA Mif2-TAF::HisMX sml1Δ::TRP, MAT a* in SCY249
HZY2461	*Cdc20-HA-AID::G418 pGPD1-OsTir1::LEU2 bar1Δ::URA Ndc80-TAF::G418 sml1Δ::TRP*, *MAT a* in SCY249
HZY2646	*Ame1-TAF::HisMX Cdc20-HA-AID::G418 pGPD1-OsTir1::LEU2 bar1Δ::URA sml1Δ::TRP, MAT a* in SCY249
HZY2777	*G418::3xFlag-Cse4 Cdc20-HA-AID::HisMX pGPD1-OsTir1::LEU2 bar1Δ::URA, sml1Δ::TRP*, *MAT a* in SCY249
HZY3059	HZE3361 in SCY249

S2 Table. Peptides identified by Data-Dependent Acquisition MS (See Supplementary Excel file)

S3 Table. Quantification of light and heavy CKP concentrations using a GST peptide standard.

Samples Analyzed	**Synthetic GST peptide: YGVSR**						**Light CKP**			**Heavy CKP**		
Cal. Levels/ Repeat	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**1**	**2**	**3**
Conc. (pM)	625	1250	2500	5000	10000	20000	14491	13881	13564	3073.3	3302.9	3427.6
Peak area (10^3)	2567	5746	10155	23086	38492	82090	59331	56842	55550	12804	13740	14247
Avg. conc. (pM)							13978.67			3267.93		
Avg. peak area (10^3)							57241			13597		
Std. dev conc.							471.15			179.72		
Std. dev peak area (10^3)							1921			732		

S4 Table. Target inclusion list for PRM-MS (See supplementary Excel file)

S5 Table. Isotope incorporation efficiencies of H-CKP

The peak areas shown are the sum of the peak areas of the top 12 transitions.

Peptide	Heavy area	Light area	% Heavy of total area
HTA2	17078343	0	100.00
CTF3	20109657	0	100.00
CBF2	19143204	1570	99.99
NKP1	22143101	20836	99.91
IML3	20163754	0	100.00
HTB2	8778000	10597	99.88
MCM21	32181955	1491	100.00
DSN1	38273619	0	100.00
MTW1	40463111	0	100.00
HHF1	24330620	0	100.00
CBF1	67708609	57839	99.91
CEP3	22579733	0	100.00
AME1	14451112	0	100.00
CHL4	44155600	62964	99.86
CTF19	27480906	1417	99.99
NDC80	26923566	0	100.00
CNN1	13304552	1245	99.99
HHT1	29087574	0	100.00
CTF13	28351430	0	100.00
MIF2-2	30827943	105111	99.66
SPC105	16052332	0	100.00
OKP1	10856738	0	100.00
NKP2*	18603646	391167*	97.94*
MIF2-1	41905182	0	100.00
CSE4	56761875	2634	100.00

* Most abundant transition (b2) is interfered with by a contaminant.

Actual isotope incorporation > 99%

S6 Table. Accuracy of calibrators (Tolerance: ± 20%):

Gray cells are outside the AMR (Tolerance: ± 20%). Cells with yellow highlights have %biases between -20% and -15% or 15% and 20%. The rest of the cells have %biases within -15% and 15%.

Peptide	Calibrator %Bias									
Ame1	18.0	5.2	-1.2	1.9	-10.0	-4.6	-3.5	-3.9	-5.0	3.0
Cbf1	7.7	9.2	-1.6	-0.7	-2.5	-0.6		-6.9	-8.7	4.1
Cbf2				-3.3	-6.0	7.2	8.9	3.3	-13.0	2.9
Cep3	3.3	3.1	-5.9	8.4	-1.2	-2.0	1.7	3.3	-15.1	4.4
Chl4	-16.3	-5.8	-4.7	3.3	5.2	10.5	11.5	0.6	-4.1	-0.2
Cnn1						15.9	-14.4	-6.2	4.7	
Cse4			11.0	-7.3	-8.4	4.6				
Ctf13		0.8	-8.0	2.1	0.2	1.0	5.8	-0.7	-1.2	
Ctf19		6.5	2.6	0.5	2.3	-12.5	3.9	-4.1		0.8
Ctf3	2.2	-1.1	-8.6	4.1	-1.8	-0.1	6.5	-0.9		-0.4
Dsn1	8.7	-1.9	-2.6	-2.5	-7.3	-1.5	6.2	1.5		-0.5
Hhf1					13.4	-7.4	-0.5	-6.7		1.2
Hht1			0.8	3.2	2.7	-2.6	-3.1	-1.0	-0.6	0.6
Hta2	0.7	0.7	-2.9	8.4	-0.1	-4.0	1.5	-5.1		0.8
Htb2				2.8	-1.6	3.1	-1.5	-2.3	-1.4	0.8
Iml3	0.4	0.1	-4.9	11.0	-3.5	2.6	-1.4	-0.3	-6.1	2.1
Mcm21	-3.9	-3.5	3.4	5.8	-1.7	-1.1	5.5	-2.0	-3.5	1.0
Mif2-1				-9.1	-1.1	4.2	1.8	5.3		-1.1
Mif2-2	14.3	-1.5	7.6	4.8	-4.3	-14.1		-8.7		2.0
Mtw1			13.7	-13.1	-4.3	3.7				
Ndc80	1.9	19.2	-9.8	-1.0	-1.9	-5.3	0.1	-4.0		0.9
Nkp1				-1.8	-6.1	9.1	5.5	2.8	-12.6	3.0
Nkp2			-8.8	-3.9	1.0	6.5	4.0	3.7	-2.2	-0.4
Okp1		-11.5	2.6	8.5	0.2	4.7	0.4	-0.8	-5.6	1.7
Spc105			1.6	-4.8	-1.3	-0.5	3.6	2.0		-0.5
Cse4 - High range						10.1	-3.4	-4.0	-4.9	2.2
Mtw1 - High range						0.1	0.1	-0.3	0.1	
Analyte conc. (pM)	**78**	**156**	**313**	**625**	**1250**	**2500**	**5000**	**10000**	**20000**	**60000**

S7 Table. Inter-day accuracy of calibrators (Tolerance: ± 20%):

Gray cells are outside the AMR (Tolerance: ± 20%). Cells with yellow highlights have average %biases between -20% and -15% or 15% and 20%. The rest of the cells have average %biases within -15% and 15%.

Peptide	Calibrator Avg. %Bias (n = 3)									
Ame1	19.5	6.8	0.3	1.8	-9.9	-1.6	-1.7	-1.6	-3.5	3.5
Cbf1	10.2	7.7	-0.7	3.1	-3.0	-5.9		0.5	-4.1	2.0
Cbf2				-7.3	-5.4	3.3	8.2	4.8	-7.6	-0.5
Cep3	2.4	8.5	2.9	7.1	1.4	-1.4	-2.5	1.7	-12.0	6.6
Chl4	-12.7	-2.1	1.6	10.5	7.1	12.8	9.4	3.5	-5.8	1.4
Cnn1						16.6	-14.2	-4.3	12.6	
Cse4			11.9	-12.1	-5.9	5.3				
Ctf13		-4.1	5.7	8.8	-0.5	6.9	1.9	0.8	-2.7	
Ctf19		9.9	4.3	-6.9	-1.5	-11.9	1.0	0.3		2.3
Ctf3	9.1	2.6	-0.2	-1.0	-0.8	-1.4	4.2	0.7		-2.0
Dsn1	2.7	-6.8	-2.6	-7.8	-3.1	-11.4	7.6	0.0		4.0
Hhf1					15.8	-7.8	-3.3	-2.3		1.5
Hht1			-9.4	10.5	-0.1	-2.3	-1.7	-3.9	-3.8	3.7
Hta2	2.3	6.5	0.4	4.6	0.3	-1.0	-1.3	-2.1		-0.7
Htb2				7.4	-3.2	1.8	2.2	2.0	-2.1	-1.2
Iml3	-10.2	-1.7	-0.3	4.5	-3.6	-0.4	-2.4	-8.3	-7.5	-0.3
Mcm21	-5.6	-2.2	1.7	5.1	-5.7	1.0	0.0	-5.7	-5.4	6.6
Mif2-1				-16.2	-5.3	-3.6	5.3	5.3		3.0
Mif2-2	7.9	-2.8	7.8	-7.0	-0.5	-13.7		-5.4		-5.2
Mtw1			15.1	-15.3	-10.4	-0.8				
Ndc80	-15.0	-6.6	-3.9	-5.2	-0.8	-9.1	6.1	-5.9		-0.4
Nkp1				-11.0	1.8	1.2	6.4	1.4	-10.3	5.0
Nkp2			-4.1	10.2	-0.8	3.2	5.5	4.7	5.2	11.0
Okp1		-18.0	0.5	11.3	1.2	3.8	2.5	-3.1	0.7	2.4
Spc105			-0.3	1.0	-4.1	-3.1	7.2	-3.1		4.8
Cse4 - High range						10.5	-3.3	-6.0	-1.8	-0.6
Mtw1 - High range						-2.5	2.2	6.1	10.7	
Analyte conc. (pM)	**78**	**156**	**313**	**625**	**1250**	**2500**	**5000**	**10000**	**20000**	**60000**

S8 Table. Inter-day precision of calibrators (Tolerance: ± 15%):

Gray cells are outside the AMR (Tolerance: ± 15%). Cells with yellow highlights have %CVs between -15% and -10% or 10% and 15%. The rest of the cells have %CVs within -10% and 10%.

Peptide	Calibrator %CV (n = 3)									
Ame1	3.6	2.9	1.7	5.8	2.6	3.2	0.7	2.4	1.0	0.6
Cbf1	4.6	5.5	5.0	2.2	0.7	5.0		4.0	2.7	6.6
Cbf2				6.5	5.0	4.8	5.3	1.8	2.2	1.7
Cep3	11.2	0.6	1.1	3.2	1.8	9.9	1.9	0.1	0.8	9.9
Chl4	8.0	6.5	7.9	3.9	5.1	6.0	2.0	1.7	1.7	1.1
Cnn1						1.3	1.4	5.1	7.7	
Cse4			2.6	0.9	2.4	4.3				
Ctf13		1.9	5.4	2.6	0.2	4.2	2.3	6.8	3.3	
Ctf19		3.1	3.2	2.3	2.5	1.3	4.7	2.4		0.5
Ctf3	14.5	4.2	2.0	6.4	5.2	2.4	4.3	3.1		4.3
Dsn1	14.1	5.0	0.8	5.2	4.7	4.1	5.9	4.2		2.6
Hhf1					2.5	1.0	2.3	1.3		2.6
Hht1			5.3	10.6	3.7	1.7	2.1	6.2	7.2	4.5
Hta2	3.4	6.0	3.7	6.8	4.7	1.9	2.4	1.6		1.0
Htb2				2.3	2.6	4.3	2.6	2.9	2.9	5.3
Iml3	8.5	4.5	1.4	4.7	9.5	2.5	2.9	4.1	3.2	0.9
Mcm21	10.8	2.2	1.4	0.1	0.4	4.1	2.7	1.2	0.5	2.6
Mif2-1				6.8	5.3	3.6	4.2	7.6		3.8
Mif2-2	8.6	7.5	2.1	5.9	7.4	5.5		5.5		3.5
Mtw1			1.9	2.2	1.0	1.5				
Ndc80	7.6	10.2	9.4	5.0	4.9	2.8	5.3	10.0		3.4
Nkp1				2.6	4.0	3.2	7.3	8.1	8.4	6.5
Nkp2			5.3	6.1	1.7	7.8	4.4	5.8	3.7	5.3
Okp1		5.8	5.2	0.9	1.7	0.5	2.3	5.5	2.8	2.8
Spc105			6.4	6.1	12.9	3.5	0.6	2.8		4.6
Cse4 - High range						2.0	1.2	1.4	1.0	0.9
Mtw1 - High range						0.9	1.0	4.2	5.1	
Analyte conc. (pM)	**78**	**156**	**313**	**625**	**1250**	**2500**	**5000**	**10000**	**20000**	**60000**

S9 Table. Quantifier and qualifier ions (L-CKP): Quantifier and qualifier transition ions for the L-CKP and corresponding ion ratios as determined from the average of the calibrators.

Peptide	Transitions				Quant/Qual ion ratio
	Quantifier	Quantifier m/z	Qualifier	Qualifier m/z	
Cbf1	T - y10++	593.7704	S - y11++	637.2864	1.33
Cep3	Y - y5+	681.3566	T - y3+	405.2092	5.62
Ctf13	D - y4+	538.2620	L - b3+	272.1605	1.10
Cse4	A - y6+	764.4301	L - y5+	693.3930	1.45
Htb2	A - b2+	209.1033	V - y6+	648.3311	1.94
Hta2	P - y4+	428.2616	F - y5+	575.3300	1.19
Hhf1	Y - y5+	695.3359	I - y6+	808.4199	1.19
Hht1	E - y5+	643.4137	L - y3+	401.2871	1.18
Mif2-1	S - y5+	545.3042	S - y7+	761.3788	1.43
Mif2-2	L - y5+	765.4042	Q - y4+	652.3202	0.58
Cbf2	N - y9+	989.4283	V - y10+	1088.4967	1.17
Mcm21	D - b3+	344.1452	D - y7+	793.3686	2.09
Ctf19	D - y6+	747.3268	S - y9+	1060.5269	2.01
Ctf3	G - y9+	958.5680	I - y4+	529.3457	0.98
Iml3	T - y4+	464.2463	V - y5+	563.3148	1.89
Chl4	P - y6+	734.4196	D - y10+	1122.5426	2.74
Mtw1	P - y11++	676.8277	L - y7+	834.4468	1.68
Cnn1	A - y2+	246.1561	L - b10+	1131.6045	2.64
Nkp1	E - y3+	417.2456	D - y7+	862.3901	0.32
Nkp2	S - y6+	704.3573	L - y4+	488.2827	1.72
Ndc80	D - y9+	1033.5273	S - y7+	831.4683	1.47
Dsn1	E - y9+	1154.4960	Y - y7+	911.4105	0.97
Spc105	Y - y5+	609.2991	S - y4+	446.2358	0.78
Okp1	Q - y5+	666.3206	A - y4+	538.2620	1.20
Ame1	D - y5+	605.3253	E - y6+	734.3679	0.81

S10 Table. Quantifier and qualifier ions (H-CKP)

Peptide	Transitions				Quant/Qual ion ratio
	Quantifier	Quantifier m/z	Qualifier	Qualifier m/z	
Cbf1	T - y10++	598.7745	S - y11++	642.2905	1.33
Cep3	Y - y5+	691.3649	T - y3+	415.2175	5.62
Ctf13	D - y4+	548.2703	L - b3+	272.1605	1.10
Cse4	A - y6+	774.4384	L - y5+	703.4013	1.45
Htb2	A - b2+	209.1033	V - y6+	658.3394	1.94
Hta2	P - y4+	438.2699	F - y5+	585.3383	1.19
Hhf1	Y - y5+	705.3442	I - y6+	818.4282	1.19
Hht1	E - y5+	653.4220	L - y3+	411.2953	1.18
Mif2-1	S - y5+	555.3125	S - y7+	771.3871	1.43
Mif2-2	L - y5+	775.4125	Q - y4+	662.3284	0.58
Cbf2	N - y9+	999.4365	V - y10+	1098.505	1.17
Mcm21	D - b3+	344.1452	D - y7+	803.3769	2.09
Ctf19	D - y6+	757.3350	S - y9+	1070.535	2.01
Ctf3	G - y9+	968.5763	I - y4+	539.3539	0.98
Iml3	T - y4+	474.2546	V - y5+	573.323	1.89
Chl4	P - y6+	744.4278	D - y10+	1132.551	2.74
Mtw1	P - y11++	681.8318	L - y7+	844.4551	1.68
Cnn1	A - y2+	256.1643	L - b10+	1131.604	2.64
Nkp1	E - y3+	427.2539	D - y7+	872.3984	0.32
Nkp2	S - y6+	714.3656	L - y4+	498.291	1.72
Ndc80	D - y9+	1043.5355	S - y7+	841.4766	1.47
Dsn1	E - y9+	1164.5043	Y - y7+	921.4188	0.97
Spc105	Y - y5+	619.3074	S - y4+	456.244	0.78
Okp1	Q - y5+	676.3288	A - y4+	548.2703	1.20
Ame1	D - y5+	615.3336	E - y6+	744.3762	0.81

S11 Table. Precision (%CV) of process replicates (n = 3) of ex vivo kinetochore reconstitutions

	%CV				
	M			G1	
Peptide	**ARS**	**CEN**	**MUT**	**CEN**	**MUT**
Ame1	19.8	3.7	21.3	24.4	30.2
Cbf1	78.0	13.3	10.4	11.7	23.4
Cbf2	6.2	4.4	20.4	34.4	
Cep3	15.4	19.4	10.3	26.8	50.5
Chl4		6.2		42.2	
Cnn1		4.4			
Cse4	5.2	7.2	6.3	43.5	
Ctf13	12.8	8.8	30.4	37.1	
Ctf19	9.6	12.9	12.0	8.9	18.9
Ctf3		13.8	20.9	53.9	31.4
Dsn1	4.0	0.7	4.7	5.6	9.5
Hhf1	10.8	10.6	17.9	28.8	44.3
Hht1	12.5	6.3	16.9	28.1	34.1
Hta2	5.7	3.1	10.4	34.9	14.4
Htb2	6.7	7.3	19.9	37.1	22.5
Iml3		15.5	23.6	36.6	41.2
Mcm21		5.0	26.9	37.2	29.0
Mif2-1		8.6			
Mif2-2	16.5	12.5		36.8	
Mtw1	10.4	4.2	11.4	18.4	36.1
Ndc80		17.9			
Nkp1		1.4			
Nkp2		40.8			
Okp1		9.1	9.8	31.9	24.2
Spc105					

%CVs were not calculated for measurements < lower limit of quantification, which are shown as empty cells in the table above.