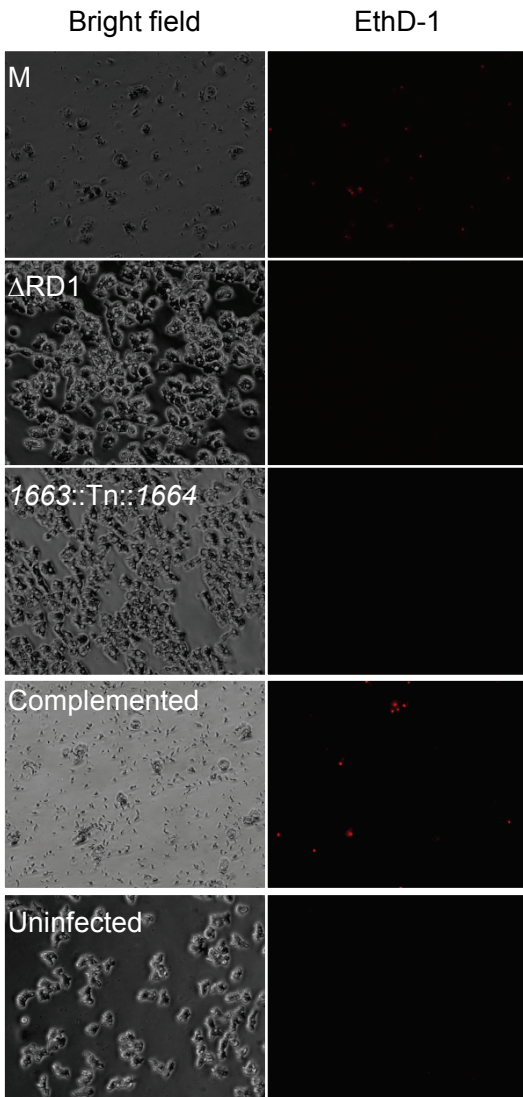
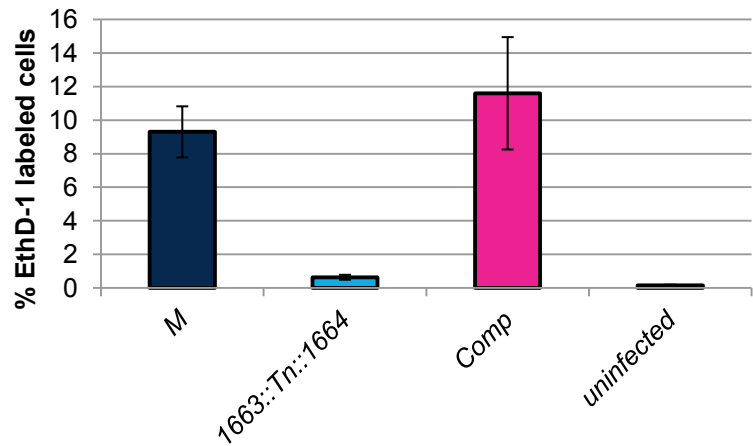


A *Acanthamoeba castellanii***B****C**

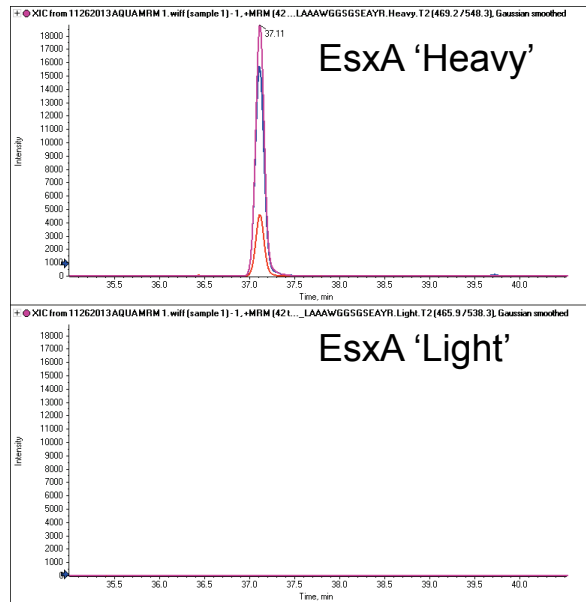
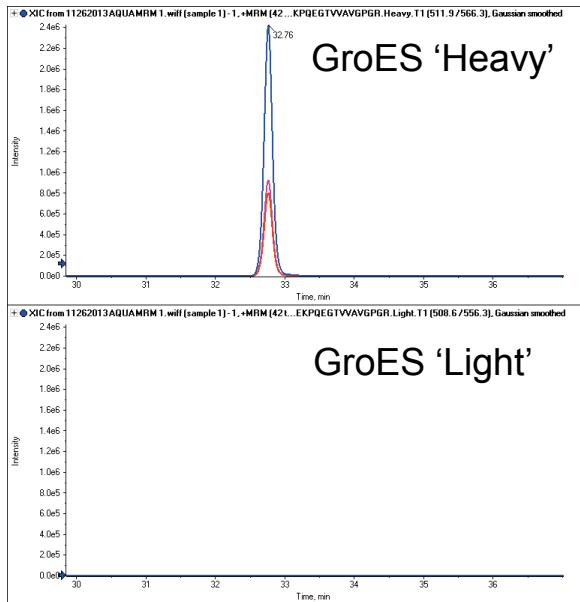
Strain	Unstained Cells	Stained Cells	Total Cells
M	220	23	243
1663::Tn::1664	1498	9	1507
Comp	1416	184	1600
Uninfected	1908	3	1911

FIGURE S1: The *MMAR_1663-1668* region of the *M. marinum* genome is required for virulence. **A. *M. marinum* infection of *A. castellanii*. **B.** Infections were performed at an MOI of 10. Images were acquired 24 hours-post infection with a 20X objective on a Zeiss Axio-Observer Microscope. Scale bars = 20 μ m. EthD-1 staining showed membrane permeabilization of the amoebae, resulting in red fluorescence. **B.** Quantitation of the average % EthD-1 stained cells per field. Red EthD1-stained cells and total cells per field were counted; 2 independent fields from each infection were counted and the counts were averaged. Error bars indicate the standard deviation between fields. **C.** Absolute counts from B, indicating the live (unstained) and the dead (stained) amoebae.**

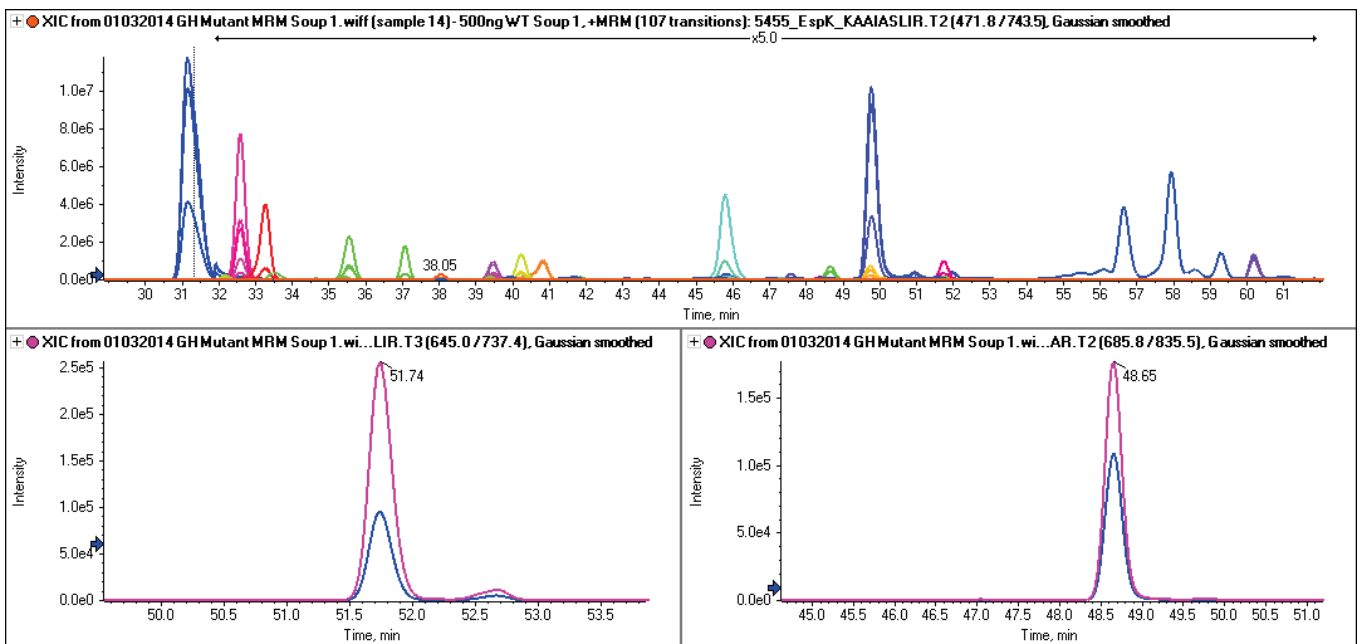
S2. List of MRM transitions used in this study

Q1 m/z	Q3 m/z	Protein_Peptide_Transition	CE (v)
571.817	943.532	CFP10_GAAGTAAQAQAVR.T1	33.6
571.817	785.463	CFP10_GAAGTAAQAQAVR.T2	33.6
571.817	714.426	CFP10_GAAGTAAQAQAVR.T3	33.6
673.336	945.49	CFP10_TQIDQVESTAGSLQAQWR.T1	38.7
673.336	688.352	CFP10_TQIDQVESTAGSLQAQWR.T2	38.7
673.336	888.469	CFP10_TQIDQVESTAGSLQAQWR.T3	38.7
827.854	999.438	CFP10_ADDEQQQALSSQMGEF.T1	46.4
827.854	886.354	CFP10_ADDEQQQALSSQMGEF.T2	46.4
827.854	840.392	CFP10_ADDEQQQALSSQMGEF.T3	46.4
829.406	915.501	ESAT6_GVQQNWDSTAQELNNSLQNLAR.T1	46.5
829.406	801.458	ESAT6_GVQQNWDSTAQELNNSLQNLAR.T2	46.5
829.406	714.426	ESAT6_GVQQNWDSTAQELNNSLQNLAR.T3	46.5
465.9	682.3	ESAT6_LAAAWGGSGSEAYR.Light.T1	20
465.9	538.3	ESAT6_LAAAWGGSGSEAYR.Light.T2	20
465.9	769.35	ESAT6_LAAAWGGSGSEAYR.Light.T3	20
469.227	692.315	ESAT6_LAAAWGGSGSEAYR.Heavy.T1	20
469.227	548.262	ESAT6_LAAAWGGSGSEAYR.Heavy.T2	20
469.227	779.347	ESAT6_LAAAWGGSGSEAYR.Heavy.T3	20
508.611	556.32	GroES_EKPQEGTWAVGPGR.Light.T1	22
508.611	869.436	GroES_EKPQEGTWAVGPGR.Light.T2	22
508.611	655.389	GroES_EKPQEGTWAVGPGR.Light.T3	22
511.944	566.32	GroES_EKPQEGTWAVGPGR.Heavy.T1	22
511.944	869.436	GroES_EKPQEGTWAVGPGR.Heavy.T2	22
511.944	665.389	GroES_EKPQEGTWAVGPGR.Heavy.T3	22
630.7	852.469	EsxN_TINYQFGDVDAHGALIR.T1	36
630.7	951.537	EsxN_TINYQFGDVDAHGALIR.T2	36
630.7	737.442	EsxN_TINYQFGDVDAHGALIR.T3	36
685.85	964.55	GROES_YGGEEYLILSAR.T1	39.3
685.85	835.5	GROES_YGGEEYLILSAR.T2	39.3
888.92	1217.58	GroEL2_DETTIVEGAGDSDAIAGR.T1	45
888.92	1118.51	GroEL2_DETTIVEGAGDSDAIAGR.T2	45
573.295	1220.62	2929_TPDAIAQDIHTTLGEK.Heavy.3T1	30
573.295	1149.59	2929_TPDAIAQDIHTTLGEK.Heavy.3T2	30
573.295	1021.53	2929_TPDAIAQDIHTTLGEK.Heavy.3T3	30
570.625	1212.62	2929_TPDAIAQDIHTTLGEK.Light.3T1	30
570.625	1141.59	2929_TPDAIAQDIHTTLGEK.Light.3T2	30
570.625	1013.53	2929_TPDAIAQDIHTTLGEK.Light.3T3	30

S3. MRM transition exemplars



Neat injection of $^{13}\text{C}^{15}\text{N}$ peptides (Cross Talk/Purity test). Shown here are chromatograms from an injection of synthesized heavy stable heavy-isotope versions of tryptic peptides from GroES (Left) and EsxA (Right). Shown on the bottom are chromatograms from the channel specific to the light (endogenous) form of EsxA and GroES. As illustrated here, a clean injection of the heavy-peptides generates no detectable signal for the corresponding light ones.



LC/MS/MS Chromatogram from WT (Culture filtrate). Expanded region to highlight peak detail (EsxB) peak on left is unscaled. Insets illustrate two typical peaks from (EsxB) left and (GroES) right.

S4. Measured MRM peak areas for cell lysate and culture filtrate analysis

Cell Lysate Data 1/1

Plt	Sample	Errors	Area Ratios	Graph Av							
	CFP10 Peptide 1		CFP10_GAAGTAAQAAVVR			ESAT6_Peptide 1		ESAT6_GVQQNWDSTAQELNNSLQNLAR.T1			
			Area/GroEL2								
	Area		Area Ratio			Area		Area Ratio			
RD1	2.58E+05	2.58E+05	1.04E+00	1.03E+00	RD1	0		0			
RD1	2.69E+05	10605.66	1.28E+00	0.2513962	RD1	0		0			
RD1	2.48E+05	4%	7.77E-01	24%	RD1	0		0			
14	2.07E+07	1.94E+07	2.76E+01	2.91E+01	14	1.39E+06	1.29E+06	2.14E+00	2.20E+00		
14	2.05E+07	2140397	3.16E+01	2.17086	14	1.20E+06	94659.04	2.05E+00	0.181698		
14	1.69E+07	11%	2.82E+01	7%	14	1.30E+06	7%	2.40E+00	8%		
Comp	2.16E+07	1.88E+07	2.30E+01	2.64E+01	Comp	1.33E+06	1.35E+06	1.80E+00	2.16E+00		
Comp	1.69E+07	2466218	2.73E+01	3.0107972	Comp	1.29E+06	65911.56	2.19E+00	0.346454		
Comp	1.79E+07	13%	2.89E+01	11%	Comp	1.42E+06	5%	2.49E+00	16%		
WT	2.34E+07	2.50E+07	3.96E+01	3.71E+01	WT	2.03E+06	2.09E+06	3.80E+00	3.60E+00		
WT	2.66E+07	1575447	3.55E+01	2.2219436	WT	2.04E+06	85912.75	3.17E+00	0.37177		
WT	2.50E+07	6%	3.61E+01	6%	WT	2.19E+06	4%	3.83E+00	10%		

	GROES_YGGEYLLSAR.T2		2929_TPDAIAQDIHTTLGK.Light.3T3		ESAT6_LAAAWGGSGSEAYR.Light.T2					
	Area		Area	Area Ratio	Area Ratio Heavy					
RD1	1.74E+07	1.59E+07	RD1	8.13E+05	7.01E+05	3.29E+00	2.95E+00	RD1	0.00E+00	
RD1	1.45E+07	1495404	RD1	6.44E+05	97228.31	3.15E+00	0.471216	RD1	0.00E+00	
RD1	1.57E+07	9%	RD1	6.46E+05	14%	2.41E+00	16%	RD1	0.00E+00	
14	1.94E+07	1.84E+07	14	1.28E+06	1.16E+06	1.97E+00	1.96E+00	14	7.06E-01	7.22E-01
14	1.90E+07	1346006	14	1.27E+06	185353.3	2.16E+00	0.206182	14	7.49E-01	0.023378
14	1.69E+07	7%	14	9.50E+05	16%	1.75E+00	11%	14	7.10E-01	3%
Comp	1.92E+07	1.81E+07	Comp	1.44E+06	1.31E+06	1.95E+00	2.08E+00	Comp	7.71E-01	7.53E-01
Comp	1.75E+07	966040	Comp	1.22E+06	117741.2	2.06E+00	0.147927	Comp	7.27E-01	0.022514
Comp	1.76E+07	5%	Comp	1.28E+06	9%	2.24E+00	7%	Comp	7.60E-01	3%
WT	1.77E+07	1.73E+07	WT	1.49E+06	1.49E+06	2.79E+00	2.56E+00	WT	1.42E+00	1.45E+00
WT	1.76E+07	689661.8	WT	1.48E+06	7023.769	2.30E+00	0.250632	WT	1.39E+00	0.082601
WT	1.65E+07	4%	WT	1.49E+06	0%	2.60E+00	10%	WT	1.55E+00	6%

Shown here are the raw peak areas (and peak area ratios) for the integrated signals from ESX-1 peptides and controls monitored in this study (Cell Lysates). Shown in green are the measured errors for the triplicate injections, in orange are the averages for each peptide/protein used in the figures.

S6. Absolute Quantification Measured MRM peak areas for cell lysate and culture filtrate analysis

				mw	Protein
ESAT6	ESAT6_LAAAWGGSGSEAYR.Heavy.T1			1404.66	9913.73
GroES	GroES_EKPQEGTVVAVGPGR.Heavy.T1			1532.81	10615.97
				3ul/tube	
			Amt (5ul) 1) Dil 1 fmol/ul	(fmol)	2) On-column
ESAT6	Stock = 142uM	710	710	2130	213
GroES	Stock = 130uM	650	650	1950	195
1) 5ul into 1000					
2) Dried, resusp to 20ul, load 2ul LC/MRM					

Transitions		
ESAT6_LAAAWGGSGSEAYR.Light		
Q1->465.9 Q3 682.3	Quantifier	
Q1->465.9 Q3 538.3	Qualifier	
Q1->465.9 Q3 769.3	Qualifier	
ESAT6_LAAAWGGSGSEAYR.Heavy		
Q1->469.2 Q3 692.3	Quantifier	
Q1->469.9 Q3 548.3	Qualifier	
Q1->469.9 Q3 779.3	Qualifier	

Culture Filtrate															
ESAT6 Light				ESAT6 Heavy				GroES Light				GroES Heavy			
Endogenous	AQUA	L/H	Endogenous	Stats	fmol /	ng per 1mg	Endogenous	AQUA	L/H	Endogenous	Stats	fmol /	ng per 1mg		
Sample Name	Area	Area Ratio	(Fmol)	Stats	500ng	filtrate	Sample Name	Area	Area Ratio	(Fmol)	Stats	500ng	filtrate		
500ng RD1 Soup 1	0.00E+00	2.16E+06	0.00E+00	0.00	Average	0.00	500ng RD1 Soup 1	7.47E+05	4.07E+07	1.84E-02	3.58	Average	3.53		
500ng RD1 Soup 1	0.00E+00	1.93E+06	0.00E+00	0.00	StDev	0	500ng RD1 Soup 1	7.46E+05	4.08E+07	1.83E-02	3.57	StDev	0.0757		
500ng RD1 Soup 1	0.00E+00	2.09E+06	0.00E+00	0.00	%CV	N/A	500ng RD1 Soup 1	7.00E+05	3.97E+07	1.77E-02	3.44	%CV	2.1%		
500ng 14 Soup 1	1.19E+04	2.02E+06	5.89E-03	1.25	Average	1.43	500ng 14 Soup 1	5.06E+06	3.81E+07	1.33E-01	25.89	Average	26.03		
500ng 14 Soup 1	1.05E+04	1.65E+06	6.38E-03	1.36	StDev	0.215	500ng 14 Soup 1	4.59E+06	3.36E+07	1.36E-01	26.58	StDev	0.4985		
500ng 14 Soup 1	1.25E+04	1.59E+06	7.83E-03	1.67	%CV	15.1%	500ng 14 Soup 1	4.18E+06	3.18E+07	1.31E-01	25.61	%CV	1.9%		
500ng C2 Soup 1	1.46E+04	1.48E+06	9.83E-03	2.09	Average	2.01	500ng C2 Soup 1	4.18E+06	3.09E+07	1.35E-01	26.41	Average	26.37		
500ng C2 Soup 1	1.23E+04	1.22E+06	1.01E-02	2.14	StDev	0.1867	500ng C2 Soup 1	3.57E+06	2.77E+07	1.29E-01	25.08	StDev	1.2693		
500ng C2 Soup 1	9.02E+03	1.07E+06	8.43E-03	1.80	%CV	9.3%	500ng C2 Soup 1	3.49E+06	2.46E+07	1.42E-01	27.61	%CV	4.8%		
500ng WT Soup 1	3.05E+06	1.14E+06	2.68E+00	571.05	Average	532.71	500ng WT Soup 1	3.86E+06	2.69E+07	1.43E-01	27.98	Average	27.59		
500ng WT Soup 1	2.97E+06	1.24E+06	2.40E+00	510.14	StDev	33.378	500ng WT Soup 1	4.10E+06	2.80E+07	1.46E-01	28.51	StDev	1.1615		
500ng WT Soup 1	3.13E+06	1.29E+06	2.43E+00	516.95	%CV	6.3%	500ng WT Soup 1	3.90E+06	2.90E+07	1.35E-01	26.28	%CV	4.2%		

Whole Cell Lysates															
ESAT6 Light				ESAT6 Heavy				GroES Light				GroES Heavy			
Endogenous	AQUA	L/H	Endogenous	Stats	fmol /	ng per 1mg	Endogenous	AQUA	L/H	Endogenous	Stats	fmol /	ng per 1mg		
Sample Name	Area	Area Ratio	(Fmol)	Stats	500ng	WC Lysate	Sample Name	Area	Area Ratio	(Fmol)	Stats	500ng	WC Lysate		
500ng RD1 Pellet 1	0	1.33E+06	0.00E+00	0.00	Average	0.00	500ng RD1 Pellet 1	4.90E+07	3.04E+07	1.61E+00	314.53	Average	296.23		
500ng RD1 Pellet 1	0	1.15E+06	0.00E+00	0.00	StDev	0	500ng RD1 Pellet 1	4.54E+07	3.02E+07	1.50E+00	292.56	StDev	16.766		
500ng RD1 Pellet 1	0	1.25E+06	0.00E+00	0.00	%CV	0%	500ng RD1 Pellet 1	4.21E+07	2.92E+07	1.44E+00	281.61	%CV	5.7%		
500ng 14 Pellet 1	8.30E+05	1.15E+06	7.21E-01	153.65	Average	158.54	500ng 14 Pellet 1	5.19E+07	3.03E+07	1.71E+00	334.08	Average	345.50		
500ng 14 Pellet 1	8.43E+05	1.18E+06	7.12E-01	151.64	StDev	10.255	500ng 14 Pellet 1	5.37E+07	3.04E+07	1.77E+00	344.31	StDev	12.049		
500ng 14 Pellet 1	7.81E+05	9.76E+05	8.00E-01	170.32	%CV	6.5%	500ng 14 Pellet 1	4.92E+07	2.68E+07	1.84E+00	358.09	%CV	3.5%		
500ng C2 Pellet 1	8.71E+05	1.26E+06	6.92E-01	147.36	Average	151.38	500ng C2 Pellet 1	5.73E+07	3.15E+07	1.82E+00	354.58	Average	354.90		
500ng C2 Pellet 1	7.73E+05	1.05E+06	7.35E-01	156.60	StDev	4.7339	500ng C2 Pellet 1	4.95E+07	2.76E+07	1.79E+00	349.14	StDev	5.9334		
500ng C2 Pellet 1	8.78E+05	1.25E+06	7.05E-01	150.19	%CV	3.1%	500ng C2 Pellet 1	4.88E+07	2.64E+07	1.85E+00	360.99	%CV	1.7%		
500ng WT Pellet 1	1.40E+06	9.56E+05	1.47E+00	312.98	Average	293.94	500ng WT Pellet 1	4.56E+07	2.48E+07	1.84E+00	358.27	Average	352.18		
500ng WT Pellet 1	1.34E+06	1.02E+06	1.32E+00	281.62	StDev	16.725	500ng WT Pellet 1	4.72E+07	2.65E+07	1.78E+00	346.93	StDev	5.7192		
500ng WT Pellet 1	1.43E+06	1.06E+06	1.35E+00	287.22	%CV	5.7%	500ng WT Pellet 1	4.65E+07	2.58E+07	1.80E+00	351.33	%CV	1.6%		

Shown here are the peak areas (and peak area ratios) for the integrated signals from ESX-1 peptides and controls monitored in this study. This includes the Area ratios of the heavy-isotope internal standards to determine absolute ratios of ESX-1 substrates.

S7. Absolute Quantification Measured MRM peak areas for colony extracts, (Surface Proteins).

				mw	Protein
ESAT6	LAAAWGGSGSEAYR.Heavy.T1			1404.66	9913.73
GroES	EKPQEGTVVAVGPGR.Heavy.T1			1532.81	10615.97
				3ul/tube	
			Amt (5ul)	1) Dil 1 fmol/ul	2) On-column
ESAT6	Stock = 142uM		710	710	2130
GroES	Stock = 130uM		650	650	1950

Transitions		
ESAT6_LAAAWGGSGSEAYR.Light		
Q1->465.9	Q3 682.3	Quantifier
Q1->465.9	Q3 538.3	Qualifier
Q1->465.9	Q3 769.3	Qualifier
ESAT6_LAAAWGGSGSEAYR.Heavy		
Q1->469.2	Q3 692.3	Quantifier
Q1->469.9	Q3 548.3	Qualifier
Q1->469.9	Q3 779.3	Qualifier

Lysis Loading Correction Factors

RD1 is 1.66of WT
 14 is 2.324 of WT
 C2 is 1.37 of WT

*Corrected to GroES, Ca 20-30ug protein obtained per colony wash

Sample Name	ESAT6 Light			ESAT6 Heavy			ESAT6 Endogenous (Fmol)	Stats	fmol / on-column	corrected ng per large colony*	Sample Name	GroES Light			GroES Heavy			GroES Endogenous (Fmol)	Stats	fmol / on-column	corrected ng per large colony*	Raw ng per large colony*
	Area	Area	Area Ratio	Area	Area	Area Ratio						Area	Area	Area Ratio	Area	Area	Area Ratio					
500ng RD1 1	0.00E+00	6.94E+05	0.00E+00	0.00	Average	0.00	0.00E+00	500ng RD1 1	1.61E+06	1.99E+07	8.11E-02	10.54	Average	10.26	1.97E+00	3.27E+00						
500ng RD1 1	0.00E+00	6.71E+05	0.00E+00	0.00	StDev	0	500ng RD1 1	1.52E+06	1.89E+07	8.03E-02	10.44	StDev	0.403368									
500ng RD1 1	0.00E+00	5.99E+05	0.00E+00	0.00	%CV	N/A	500ng RD1 1	1.45E+06	1.92E+07	7.54E-02	9.80	%CV	3.9%									
500ng 14 1	3.24E+05	5.33E+05	6.08E-01	86.29	Average	81.43	1.04E+01	500ng 14 1	1.81E+06	1.76E+07	1.03E-01	13.41	Average	14.37	1.97E+00	4.58E+00						
500ng 14 1	2.59E+05	4.98E+05	5.20E-01	73.88	StDev	6.624001	500ng 14 1	1.70E+06	1.41E+07	1.21E-01	15.70	StDev	1.1898182									
500ng 14 1	3.03E+05	5.12E+05	5.92E-01	84.11	%CV	8.1%	500ng 14 1	1.74E+06	1.61E+07	1.08E-01	13.98	%CV	8.3%									
500ng C2 1	2.96E+05	5.26E+05	5.64E-01	80.05	Average	82.58	1.79E+01	500ng C2 1	1.17E+06	1.67E+07	7.02E-02	9.12	Average	8.47	1.97E+00	2.70E+00						
500ng C2 1	3.01E+05	5.54E+05	5.43E-01	77.05	StDev	7.136588	500ng C2 1	1.11E+06	1.65E+07	6.75E-02	8.78	StDev	0.8389124									
500ng C2 1	9.98E+03	1.56E+04	6.38E-01	90.63	%CV	8.6%	500ng C2 1	4.50E+03	7.77E+04	5.79E-02	7.53	%CV	9.9%									
500ng WT 1	4.50E+05	7.45E+05	6.04E-01	85.75	Average	79.33	2.37E+01	500ng WT 1	9.56E+05	2.11E+07	4.54E-02	5.90	Average	6.18	1.97E+00	1.97E+00						
500ng WT 1	3.95E+05	7.26E+05	5.44E-01	77.22	StDev	5.138667	500ng WT 1	9.63E+05	2.07E+07	4.64E-02	6.03	StDev	0.374591									
500ng WT 1	3.85E+05	7.14E+05	5.39E-01	76.53	%CV	6.4%	500ng WT 1	9.67E+05	1.90E+07	5.08E-02	6.61	%CV	6.1%									

Shown here are the peak areas (and peak area ratios) for the integrated signals from ESX-1 peptides and controls monitored in this from colony extracts. This includes the Area ratios of the heavy-isotope internal standards to determine absolute ratios of ESX-1 substrates. Shown in peach are the correction factors used for GroES (Confirmed with GroEL) used to normalize protein response between the colonies.