Towards Comprehensive Signaling Pathway Monitoring Using Advanced PRM Methods

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ABSTRACT

Purpose: Evaluation of the performance of advanced parallel reaction monitoring (PRM) methods to monitor signaling pathways in cancer cell lines for clinical research.

Methods: A broad range of PRM methods (including internal standard triggered-PRM[1,2]; IS-PRM), corresponding to various scale format implementations on different quadrupole-Orbitrap instrument generations (Thermo Scientific™ Q Exactive™ HF MS and new Thermo Scientific™ Q Exactive™ HF-X MS), were evaluated, based on targeted proteomics experiments applied to signaling pathways.

Results: Recent technology developments, offered by the new Q Exactive HF-X mass spectrometer and the most advanced acquisition methods, provided substantial improvement in the scale and data quality of targeted quantification experiments. They allowed significant increase in depth and breadth of coverage in signaling pathway monitoring.

INTRODUCTION

Targeted mass spectrometry quantification based on high-resolution and accurate-mass (HRAM) parallel reaction monitoring (PRM) measurements is increasingly being adopted by the proteomics community. The PRM technique offers unmatched degrees of selectivity and analytical sensitivity, typically required to analyze peptides in complex samples. Combined with advanced acquisition schemes, *e.g.*, internal standard triggered-PRM[1,2] (IS-PRM), the technique benefits from further improvement in experiment scale, data quality, workflow automation, and robustness. Here the performance of PRM has been revisited to evaluate the advantages enabled by the latest technology developments offered by the new Q Exactive HF-X mass spectrometer and the most advanced acquisition methods. The new state-of-the-art methods were applied to the wide targeted profiling of signaling pathways.

Several signaling pathways were monitored (including MAPK, WnT, RAS, ErbB, and AKT/mTOR pathways) with the intent to achieve the deepest and broadest coverage at the levels of proteins, peptides, and post-translational modifications (*e.g.*., phosphorylation). They play a central role in tumor progression and anti-cancer drug resistance. Comprehensive knowledge about the expression of their protein components, including post-translational modifications, is thus vital to cancer research.

MATERIALS AND METHODS

Sample Preparation

Proteins were extracted from human HeLa, HCT116, A549, MCF7, HEK293, and yeast cells. After reduction and alkylation, proteins were digested with trypsin.
A set of 80 high-purity Pierce™ SIL peptides corresponding to 39 proteins was spiked at 20 fmol/µL in 250 ng/µL of cancer cell protein digest (HeLa, HCT116, A549, MCF7, and HEK293) and used to follow AKT/mTor and RAS pathways in moderate-scale experiments.
A set of 30 standard-purity synthetic unlabeled Pierce™ peptides corresponding to 13 signaling pathway proteins was spiked in various calibrated amounts in 250 ng/µL of yeast protein digest supplemented with SIL forms of the peptides at 20 fmol/µL. The dilution curve spanned a concentration range from 0.8 to 500 amol/µL.
A set of 500 or 650 standard-purity SIL peptides (Pierce, Rockford, IL; JPT Peptide Technologies, Berlin, Germany) corresponding to 93 signaling pathway proteins was spiked at 20 fmol/µL (nominal concentration) in 250 ng/µL of cancer cell protein digest (HeLa, HCT116, A549, MCF7, and HEK293) and used to follow MAPK, WnT, RAS, ErbB, and AKT/mTOR pathways in large-scale experiments.
All samples were supplemented with a mixture of peptides (at 30 fmol/µl (elution time landmarks, PRTC peptides, PN 88321, Pierce, Rockford, IL). One µL of each sample was injected for LC-MS/MS analyses.

Methods

Chromatographic separations were performed over a one-hour gradient on a Thermo Scientific™ EASY-nLC™ 1000 system equiped with a Thermo Scientific™ PepMap™ C_{18} analytical column (2 µm, 100 Å, 0.075 x 150 mm). The LC system was coupled to Q Exactive HF and Q Exactive HF-X quadrupole-Orbitrap instruments. Peptide LC monitoring window widths in time-scheduled PRM analyses were set at 2.5 min, based on previously observed middle-term stability of peptide elution times.

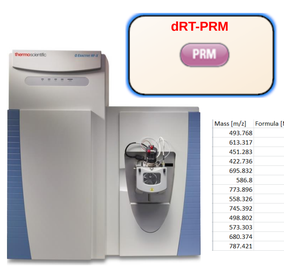
Mass spectrometers were operated with a broad range of PRM methods, including IS-PRM (using the instrument application programming interface, iAPI). Under its main implementation, the IS-PRM technique alternated between i) a "watch mode", in which internal standards (IS) were continuously measured in their (dynamically corrected) elution time monitoring windows at fast scanning rates, and ii) a "quantitative mode" (triggered by the real-time detection of the IS by means of spectral matching), which measured the corresponding pairs of IS and endogenous peptides serially over their elution profile, using optimized acquisition parameters (Figures 1 and 2). A variant of the IS-PRM method, relying on the simultaneous measurement of SIL and endogenous peptides by "broadband" acquisition of a single MS/MS spectrum, was developed and tested (Figure 2). Figure 2 also displays the acquisition methods an parameter settings utilized in the various IS-PRM experiments.

Figure 1. Data acquisition scheme of IS-PRM[1,2].

A) On-the-fly correction of peptide monitoring windows

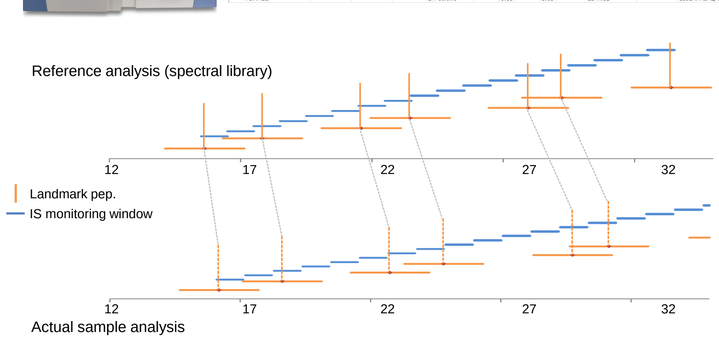
The first step of IS-PRM acquisition process has been implemented as a standalone method (dRT-PRM) in the standard method editor of the latest version of Q Exactive controlling software (Exactive Series ICSW Tune 2.9, requires the addition of PRTC peptides to all samples).

Principle:
- Determination of the elution times of IS and landmark peptides in reference analyses.
- Capture in real-time of the elution times of landmark peptides in actual sample analyses.
- On-the-fly correction of the monitoring windows of IS not yet eluted.



PRTC peptides are included in PRM experiments

Mass [m/z]	Formula [M]	Formula type	Species	CS [z]	Polarity	Start [min]	End [min]	(N)CE	(N)CE type	MSX ID	Comment
493.768				2	Positive	11.92	14.92	25	NCE		SSAAPPPPPR[HeavyR]
613.317				2	Positive	14.98	16.98	25	NCE		GISNEGQNASIK[HeavyK]
451.283				2	Positive	18.52	20.52	25	NCE		DIPVPKPK[HeavyK]
422.736				2	Positive	21.32	23.32	25	NCE		IGDYAGIK[HeavyK]
695.832				2	Positive	26.06	28.06	25	NCE		TASEFDSAIAQDK[HeavyK]
586.8				2	Positive	27.5	29.5	25	NCE		SAAGAFGPELSR[HeavyR]
773.896				2	Positive	31.53	33.53	25	NCE		ELGQDGVDTYLQTK[HeavyK]
558.326				2	Positive	35.19	37.19	25	NCE		GLILVGGYGTR[HeavyR]
745.392				2	Positive	36.15	38.15	25	NCE		SFANQPLEVVYSK[HeavyK]
498.802				2	Positive	39.69	41.69	25	NCE		LTILEELR[HeavyR]
573.303				2	Positive	42.57	44.57	25	NCE		NGFILDGFPR[HeavyR]
680.374				2	Positive	44.22	46.22	25	NCE		ELASGLSFPVGFK[HeavyK]
787.421				2	Positive	46.65	48.65	25	NCE		LSSEAPALFQFDLK[HeavyK]



B) Measurement of endogenous peptide triggered by SIL peptide detection

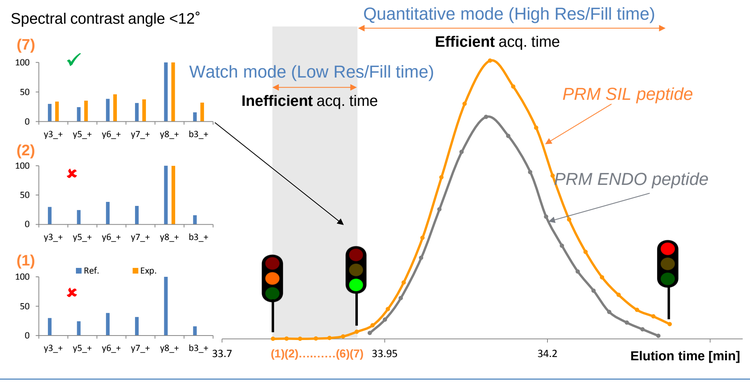
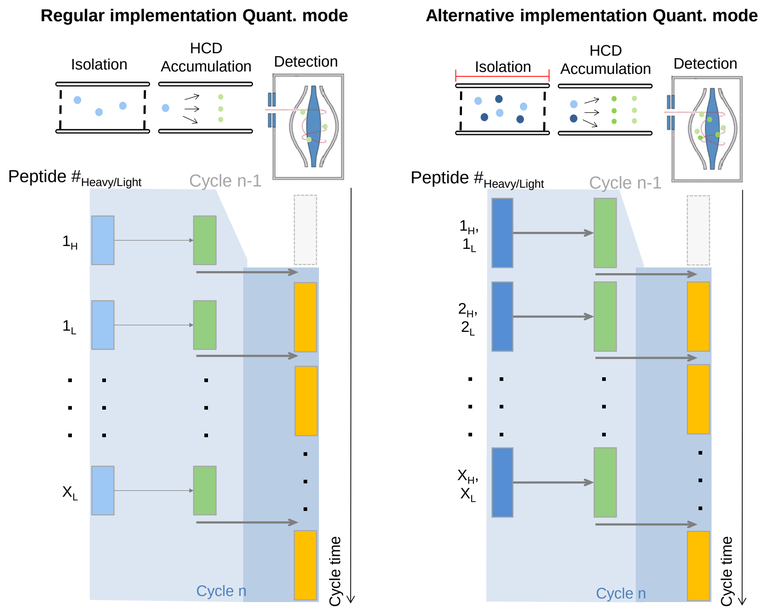


Figure 2. Acquisition methods and parameters of IS-PRM.



"Sequential" measurement of SIL/ENDO
Multiple cycle acquisition

"Broadband" measurement of SIL/ENDO
Single cycle acquisition

MS Settings	*SIL Peptide Measurement*			*ENDO Peptide Measurement*		
	Fill time	Resolution	Acquisition rate	Fill time	Resolution	Acquisition rate
QE Plus	41 ms	17.5k	14 Hz	110-500 ms	35-140k	7 Hz
QE HF	20 ms	15k	20 Hz	110-500 ms	60-240k	2-7 Hz
LC window	***Watch Mode***			***Quant Mode***		
	SIL		ENDO	SIL		ENDO
"Sequential"	≈ 0.3 min (18 s)		-	≈ 0.4 min (24 s)		≈ 0.4 min (24 s)
"Broadband"	≈ 0.3 min (18 s)		-	< 0.05 min (< 3 s; 1 cycle)		

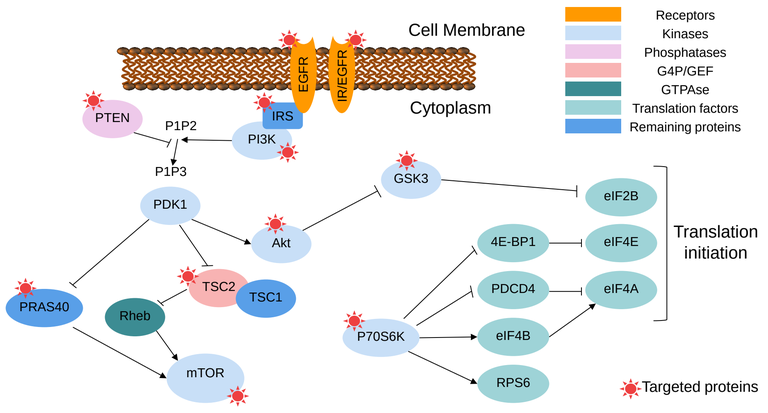
RESULTS

Signaling Pathway Monitoring by PRM and IS-PRM

Moderate-scale analysis of AKT/mTOR and RAS pathways

The initial moderate-scale experiment aimed at the monitoring of the protein components of the Akt/mTOR and RAS pathways in five cancer cell line digests (HeLa, HCT116, A549, MCF7, HEK293). A total of 80 pairs of SIL and endogenous peptides were targeted in PRM and regular (sequential) IS-PRM analyses on a Q Exactive HF MS. A subset of these targets (30 SIL peptides corresponding to 13 proteins from AKT/mTOR pathway, Figure 3) was included in the measurement of the dilution series of corresponding 30 unlabeled synthetic peptides in yeast digest for the determination of their limit of quantification (LOQ) in PRM and IS-PRM analyses. The acquisition parameters in these two experiments were adjusted, taking into account the number of peptides included and their elution time, to maintain a cycle time below 2.5-3 s over the entire LC separation, and thus ensure sufficient sampling rate. For the analyses of cancer cell lines, this resulted in the use of an Orbitrap resolution of 30k and 120k, and a maximum fill time of 50 and 250 ms in PRM and IS-PRM analyses ("quantitative" mode), respectively. The lower number of peptides included in dilution series analyses enabled a two-fold increase in acquisition time.

Figure 3. Proteins targeted in AKT/mTOR pathway



The quantification performance of the two techniques was compared. The higher quality of data generated by IS-PRM analyses was reflected in dramatically increased sensitivity (5-fold increase as compared with PRM) in dilution series measurements (Figure 4). This also translated in far better endogenous peptide detection success (> 50% increase, up to 64 peptides) in the analyses of the five cancer cell lines (Figure 5), while maintaining high quantification reproducibility (coefficient of variation below 5% in the vast majority of quantification points, Figure 6).

Figure 4. Limits of quantification of peptides from dilution series analyses.

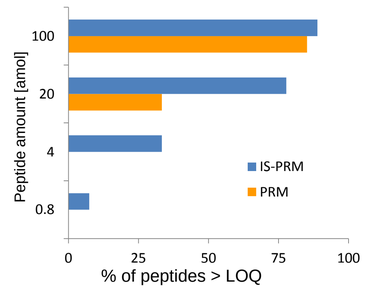


Figure 5. Endogenous peptides detected in the analyses of the cell line digests.

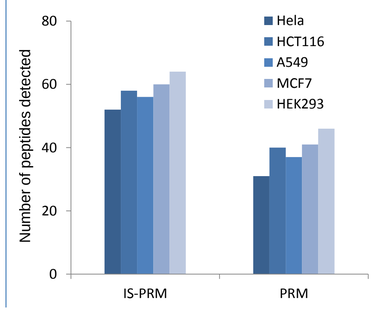
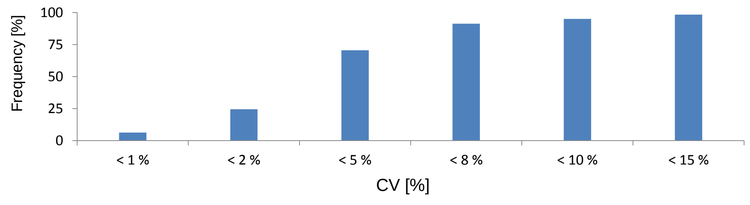


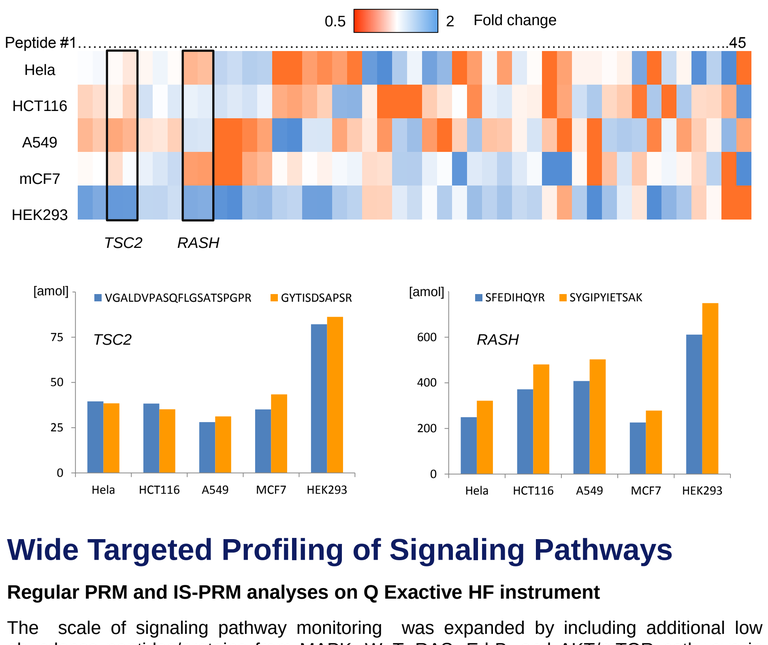
Figure 6. Quantification reproducibility in triplicated IS-PRM analyses of the 80 pairs of SIL and endogenous peptides in the five cancer cell line digests.



Accurate quantification of AKT/mTOR and RAS pathway components in the cancer cell lines

The heat map, prepared from the 45 endogenous peptides systematically quantified in the five cancer cell lines by IS-PRM analyses, reflects the magnitude of their abundance change (Figure 7, upper panel). The relative quantification results obtained for the surrogate peptides of common proteins were well correlated and revealed significant changes in protein regulation over the different cell lines. Spiking IS in calibrated amounts allowed accurate quantification of peptides, which exhibited acceptable consistency at the protein level (Figure 7, lower panel).

Figure 7. Quantification of pathway components in cancer cell lines. Relative quantification results are displayed in upper panel. Accurate quantification results were extracted for two proteins in the lower panel.

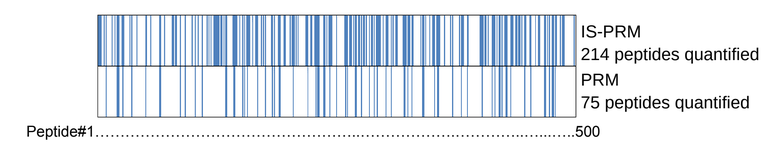


Wide Targeted Profiling of Signaling Pathways

Regular PRM and IS-PRM analyses on Q Exactive HF instrument

The scale of signaling pathway monitoring was expanded by including additional low abundance peptides/proteins from MAPK, WnT, RAS, ErbB, and AKT/mTOR pathways in order to operate slightly above scale limit of PRM method. This large-scale experiment included 500 pairs of SIL and endogenous peptides, corresponding to 93 proteins. The acquisition parameters were adjusted accordingly, resulting in the use of a resolution of 15 and 60k, and a maximum fill time of 20 and 110 ms in PRM mode and quantitative mode of IS-PRM, respectively. A total of 214 endogenous peptides were quantified by IS-PRM in Hela cell digest, allowing a three-fold increase in coverage depth as compared with PRM analyses in which only 75 peptides were quantified (Figure 8).

Figure 8. Endogenous peptides quantifiable in Hela cell sample (Q-Exactive HF)



Advanced PRM methods operated on Q Exactive HF-X instrument

The new Q Exactive HF-X MS offers several features beneficial to PRM analyses, among which a significant reduction in scan time overhead, enabling more sensitive measurements at the fastest MS/MS acquisition rate. Large-scale signaling pathway monitoring in Hela cell sample was reproduced and even expanded to 650 pairs of SIL and endogenous peptides by leveraging this feature to maximize the scanning rate to 40 Hz in the PRM mode and the watch mode of IS-PRM (Figure 9). It was also combined with a new variant of IS-PRM, relying on the acquisition of a single MS/MS spectrum to measure simultaneously each pair of IS and endogenous peptides, through broadband quadrupole isolation, in quantitative mode (detailed in Figure 9). By contrast with the regular (sequential) IS-PRM method, still using same acquisition parameters in quant. mode, the performance of PRM method, which operated close to its scale limit, suffered from the low resolution and fill time used. This was reflected by only 32 endogenous peptides quantified (vs. 274 peptides in IS-PRM analysis. The variant of IS-PRM method provided acceptable quantification results (120 peptides), while still operating far below its scale limit. In comparison with regular IS-PRM, its measurements suffer from a drop in dynamic range resulting from the broadband isolation window (7 Th). The scale and sensitivity capabilities of the different methods were summarized in Table 1.

Figure 9. Endogenous peptides quantifiable in Hela cell sample (Q-Exactive HF-X)

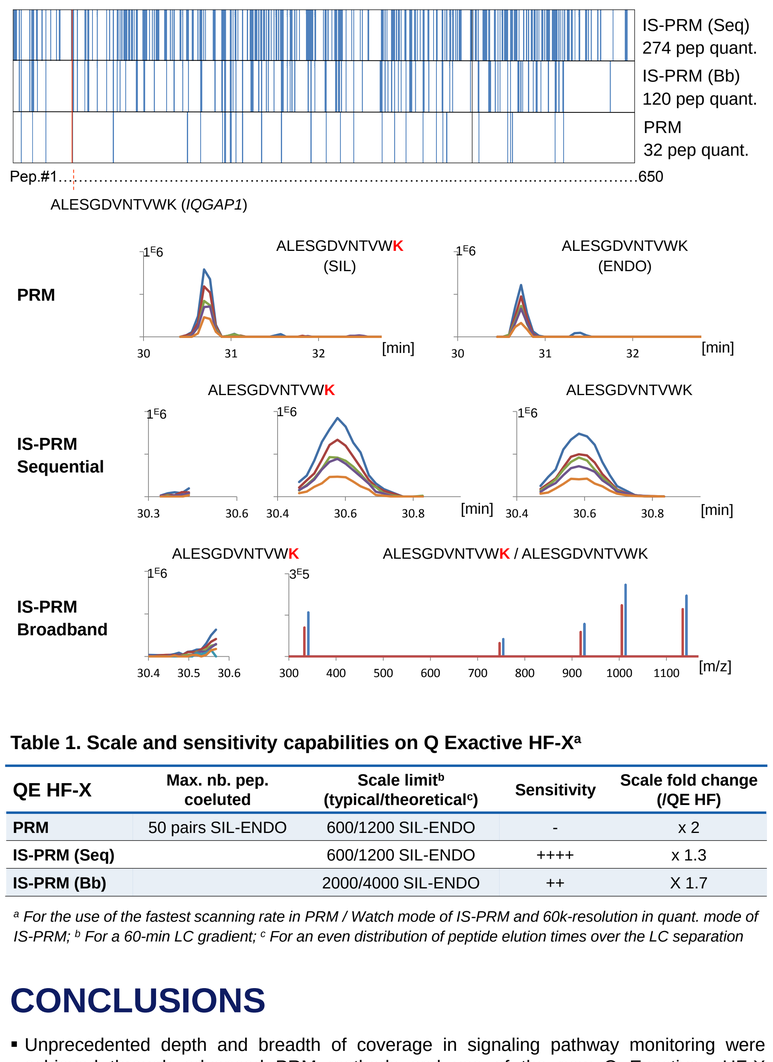


Table 1. Scale and sensitivity capabilities on Q Exactive HF-X[a]

QE HF-X	Max. nb. pep. coeluted	Scale limit[b] (typical/theoretical[c])	Sensitivity	Scale fold change (/QE HF)
PRM	50 pairs SIL-ENDO	600/1200 SIL-ENDO	-	x 2
IS-PRM (Seq)		600/1200 SIL-ENDO	++++	x 1.3
IS-PRM (Bb)		2000/4000 SIL-ENDO	++	X 1.7

[a] For the use of the fastest scanning rate in PRM / Watch mode of IS-PRM and 60k-resolution in quant. mode of IS-PRM; [b] For a 60-min LC gradient; [c] For an even distribution of peptide elution times over the LC separation

CONCLUSIONS

- Unprecedented depth and breadth of coverage in signaling pathway monitoring were achieved through advanced PRM methods and use of the new Q Exactive HF-X instrument.
- The IS-PRM method relying on broadband isolation and single spectrum acquisition showed the highest scale capabilities but requires additional refinement to address dynamic range limitations.

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REFERENCES

1. Gallien S, Kim SY, and Domon B; *Mol. Cell. Proteomics*, 2015
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TRADEMARKS/LICENSING