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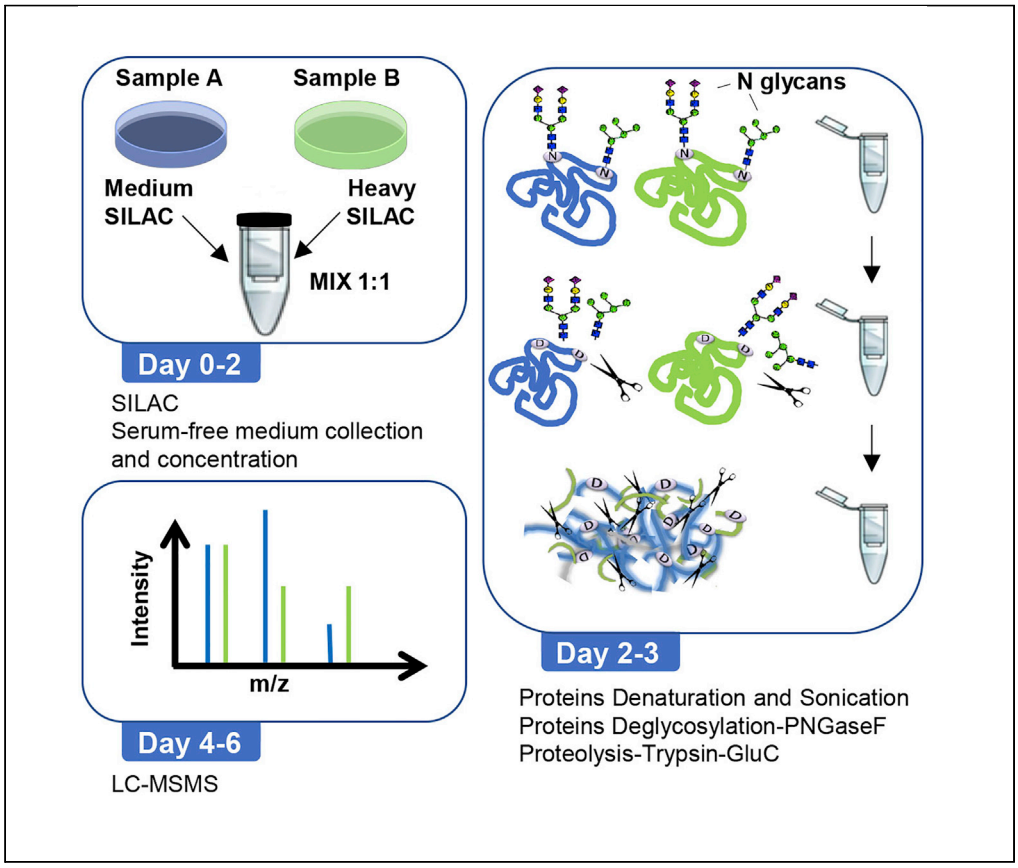
Secret3D Workflow for Secretome Analysis



Secretome analysis is crucial to unravel extracellular processes. However, secreted proteins are difficult to detect in mass-spectrometry-based analysis due to contamination of serum proteins deriving from cell culture media and to high glycosylation, which hampers tryptic digestion. Secret3D workflow is an optimized protocol for the global analysis of secretome from *in vitro* cultured cells. It allows efficient and robust protein identification and quantitation and provides information on putative N-glycosylation sites of the secreted proteins.

Vittoria Matafora, Angela Bachi

vittoria.matafora@ifom.eu (V.M.)
angela.bachi@ifom.eu (A.B.)

HIGHLIGHTS

Secret3D workflow allows the analysis of secreted proteins from *in vitro* cultured cells

De-glycosylation and double digestion enhance protein identification and quantification

Identification of putative glycosylation sites of the secreted proteins

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Secret3D Workflow for Secretome Analysis

Vittoria Matafora[1,2,*] and Angela Bachi[1,3,*]

[1]IFOM- FIRC Institute of Molecular Oncology, 20139 Milan, Italy

[2]Technical Contact

[3]Lead Contact

*Correspondence: vittoria.matafora@ifom.eu (V.M.), angela.bachi@ifom.eu (A.B.)
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SUMMARY

Secretome analysis is crucial to unravel extracellular processes. However, secreted proteins are difficult to detect in mass-spectrometry-based analysis due to contamination of serum proteins deriving from cell culture media and to high glycosylation, which hampers tryptic digestion. Secret3D workflow is an optimized protocol for the global analysis of secretome from *in vitro* cultured cells. It allows efficient and robust protein identification and quantitation and provides information on putative N-glycosylation sites of the secreted proteins.

For complete details on the use and execution of this protocol, please refer to Matafora et al. (2020).

BEFORE YOU BEGIN

Prepare Medium and Heavy Cell Media for Cell Culture

Timing: can be made before starting cell culture

1. Use Dulbecco Modified Eagle's Medium DMEM deprived of arginine and lysine + 5% FBS Dialyzed + 2 mM L-Glutamine or RPMI-1640 deprived of arginine and lysine + 10% FBS Dialyzed + 2 mM L-Glutamine according to cell type and supplemented with medium- and heavy-labeled amino acids, added at the concentration originally contained in the media. The medium culture contains arginine (L-Arg $^{13}C_6$-$^{14}N_4$) and lysine (L-Lys-4,4,5,5-D4) and the heavy culture contains arginine (L-Arg $^{13}C_6$-$^{15}N_4$) and lysine (L-Lys $^{13}C_6$-$^{15}N_2$) amino acids (Ong et al., 2002).

Incorporate Medium- and Heavy-Labeled Amino Acids into the Proteome of Cultured Cells

Timing: 1–2 weeks

2. Cells are cultured in the labeled medium for at least five cell divisions to obtain full incorporation of the labeled amino acids in their proteome.

Perform Incorporation Test

Timing: 1 day

3. Analyze the proteome of a small aliquot of cell lysate to check the level of incorporation of labeled arginine and lysine (Harsha et al., 2008).

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⚠ CRITICAL: The incorporation level should be at least 95%, otherwise the quantitation is biased toward the light condition. If the cells are not fully incorporated, the culture in medium and heavy cell media should be continued until full incorporation is reached.

Starve the Cells in Serum-free Medium

Timing: 1 day

4. Equal numbers of cells are split into 15 cm dishes at roughly 50% confluence. Once cell lines reach ~70% confluence, one 15-cm dishes of each cell line is washed 3× with PBS and 3× with serum-free media. Cells are starved in serum-free medium for 18 h.

Check Cells Viability upon Serum Starvation

Timing: 2 h

5. Collect the medium for the analysis of the secreted proteins (secretome). The remaining adherent cells are collected by trypsinization and used for viability assay.

⚠ CRITICAL: Viability ≥95% is accepted, avoid dead cells that might contaminate the secretome.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Heavy L-arginine-$^{13}C_6$,$^{15}N_4$ HCl	Isotec (Sigma)	Cat#608033-500 MG
Heavy L-lysine-$^{13}C_6$,$^{15}N_2$ 2HCl	Cambridge Isotope Laboratories	Cat#CILCLM-291-0.25
Medium L-arginine-$^{13}C_6$ HCl	Cambridge Isotope Laboratories	Cat#CILCLM-2265-05
Medium L-lysine-4,4,5,5-D_4 2HCl	Cambridge Isotope Laboratories	Cat#CILCLM-2640-0.5
2-Chloroacetamide	Sigma-Aldrich	Cat#C0267
Ammonium bicarbonate	Sigma-Aldrich	Cat#09830
Acetonitrile	Fisher Chemical	Cat#A955-4
Formic acid	Fisher Chemical	Cat#A117-50
Tris hydrochloride Tris-HCl	Sigma-Aldrich	Cat#10812846001
Sodium chloride	Sigma-Aldrich	Cat#S7653-250G
Phosphate buffered saline PBS	Sigma-Aldrich	Cat#P5493
TCEP	Thermo scientific	Cat#20490
Buffer G	New England Biolabs	Cat#P0704S
PNGase F	New England Biolabs	Cat#P0704S
Trypsin, Sequencing Grade, modified	ROCHE	Cat#11 418 025 001
Endoproteinase Glu-C Sequencing Grade	ROCHE	Cat#11420399001
DMEM, high glucose, no glutamine, no lysine, no arginine	Thermo Fisher Scientific	Cat#A1443101
RPMI Medium (lysine and arginine depleted)	Thermo Fisher Scientific	Cat#88365
FBS Dialyzed USA Origin	Invitrogen	Cat#26400044

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Bradford	Applied Biosystems	Cat#4368814
Deposited Data		
.RAW files were deposited together with all peptides identified and parameters used for the analysis	Peptide Atlas repository	http://www.peptideatlas.org/PASS01358
Experimental Models: Cell Lines		
IGR37	DSMZ	Cat#ACC-237
IGR39	DSMZ	Cat#ACC-239
Software and Algorithms		
Microsoft Excel 2010	Microsoft Office	N/A
MaxQuant	Max Planck Institute of Biochemistry	https://www.maxquant.org/maxquant/
Perseus	Max Planck Institute of Biochemistry	https://www.maxquant.org/perseus/

MATERIALS AND EQUIPMENT

Tris 100 mM buffered to pH 8.0 (Tris-HCl), urea 8 M is required. This buffer is for the solubilization of secreted proteins.

TCEP solution 1 M and chloroacetamide 1 M in Tris-HCl are required to reduce and alkylate disulfide bonds of cysteine residues.

Ammonium bicarbonate 50 mM is required to solubilize trypsin, phosphate buffer pH 7.8 is used to solubilize Glu-C, while NaCl 0.5 M solution is required to elute peptides from the filter.

Solvent A (2% ACN, 0.1% formic acid) and solvent B (80% ACN, 0.1% formic acid) are required for nanoLC separation of the purified peptides.

The Bradford Assay can be used for protein quantification, but any other similar methods could also be used.

For mass spectrometry analysis, high-resolution Q-Exactive HF mass spectrometer connected to the EASY-nLC 1000 HPLC system (Thermo Fisher Scientific) allows the proteomic analysis of secreted proteins, but also other high-resolution mass spectrometers could be used.

Data analysis software required: Xcalibur 4.0, MaxQuant 1.6, Perseus 1.5. MaxQuant software (1.5.2.8) and Perseus software (version 1.5.6.0) are used for the analysis showed below; however, other versions are suited for the quantitative analysis.

STEP-BY-STEP METHOD DETAILS

Seeding Cells (Day 0)

Timing: approximately 30 min

This step describes how to seed the medium- or heavy-labeled IGR37 or IGR39 melanoma cell line.

1. Remove and discard culture medium from the pre-cultured IGR37 or IGR39 melanoma cells in 15 cm dish.

2. Wash attached cells with 10 mL of autoclaved phosphate buffered saline (PBS) for three times to remove serum (Troubleshooting 1).
3. Add 1.0 mL of 0.05% Trypsin-EDTA solution, incubate at 37°C for 3–5 min and observe cells under an inverted microscope. If required, extend the incubation time until all the cells detach from the plastic dish.
4. Add 9.0 mL of complete medium- or heavy-labeled growth medium and aspirate cells by gently pipetting.
5. Transfer the cell suspension into a 15 mL conical centrifuge tube and centrifuge at 250 × *g* for 5 min at 25°C (Troubleshooting 2).
6. Remove and discard culture medium and suspend the cell pellet in 1.0 mL complete medium- or heavy-labeled growth medium and check the cell number using a cell counter.
7. Seed 1 × 10^6 cells in 10 mL complete medium- or heavy-labeled growth medium into one 15 cm dish.

Note: generally, by seeding 1 × 10^6 cells more than 2 × 10^6 cells are collected at day 2. If not, seed more than one plate for each sample.

Note: depending on the cell type, 1 × 10^6 cells may look sparse, in this case seed at least at 50% confluency.

Cells Starvation in Serum-free Medium (Day 1)

Timing: 30 min

This step describes how to eliminate serum proteins from the medium.

8. Remove and discard the culture medium.
9. Wash the attached cells with 10 mL of autoclaved PBS for three times.
10. Wash the attached cells with 10 mL of medium- or heavy-labeled growth medium deprived of fetal bovine serum (FBS) for three times.
11. Add 10 mL of medium- or heavy-labeled growth medium FBS deprived, starve the cells for 18 h.

Secretome Preparation (Day 2)

Timing: 3 h

This step describes how to prepare the secreted proteins extract.

12. Collect the medium from 2 × 10^6 cells previously starved in serum-free medium for 18 h. Centrifuge at 250 × *g* for 5 min to eliminate dead cells. Filter the supernatant with 0.22 μm filters.
13. Mix the heavy and medium secretomes 1:1. Concentrate the secreted proteins by using microcon filters with 10 kDa cutoff (Millipore) (capacity 15 mL) until 500 μL. Centrifuge at 4,500 × *g* for 10 min (Figure 1A).

Note: if the volume of the combined secretomes exceeds the microcon capacity, load the sample in steps of 15 mL each until the entire volume is loaded on the filter.

14. Denature proteins by changing the buffer with urea 8 M Tris-HCl 100 mM at pH 8 (urea buffer) as follows. Add urea buffer on the top of the microcon filter, centrifuge at 9,300 × *g* for 10 min and discard the flow through. Perform at least three washes with 5 mL of urea buffer or until the solution become transparent. Transfer the secreted protein extract (generally about 500 μL) into an Eppendorf tube and sonicate with BIORUPTOR (3 cycles: 30 s on/30 s off).

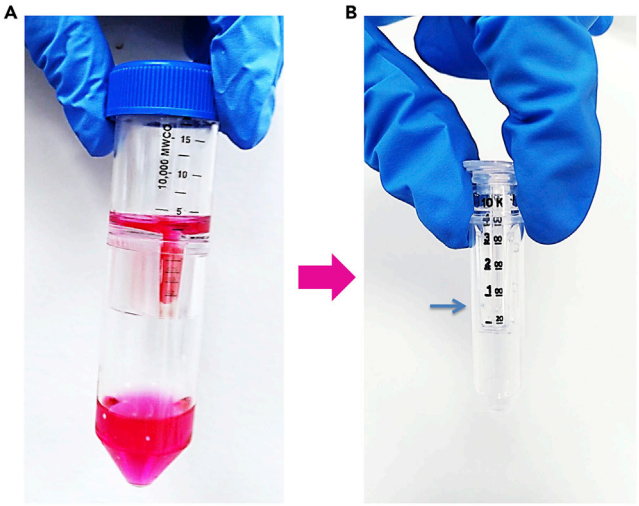

Figure 1. Concentration and Buffer Exchange of Secreted Proteins

(A) Concentration of the cell culture medium. The solution in the lower part of the Falcon tube is discarded, while secreted proteins, present in the upper part, are concentrated to 500 μL.

(B) Further concentration of the secretome. The solution at the bottom of the tube is discarded, while secreted proteins, present in the upper part of the filter and indicated by the arrow, are concentrated to 30 μL.

Note: the sonication step is important to destroy proteins aggregates and to release proteins from extracellular vesicles allowing proteins to be properly solubilized.

15. Quantify proteins concentration by using Bradford assay. Troubleshooting 3

Note: in our experiments, 2 × 10^6 cells produce more than 30 μg of secreted proteins. This amount is sufficient for the MS analysis.

Pause Point: The secreted protein extract can be stored at −80°C for at least several weeks.

Secretome De-glycosylation and Proteolytic Digestion (Day 2)

Timing: 2 days

This step describes how to reduce and alkylate cysteines of secreted proteins and how to perform proteins de-glycosylation and double digestion.

16. By using smaller (about 400 μL capacity) microcon filters with 10 kDa cutoff (Millipore), reduce the volume of 30 μg of secreted proteins to 30 μL. Add the secreted proteins on the top of the filter (Figure 1B). Centrifuge 10 min at 9,300 × *g* at 25°C. Discard the flow through. As the secretome will be more than 400 μL, load the secreted proteins in steps of 400 μL each until the entire sample is loaded. Leave the proteins on the filter and perform cysteines reduction and alkylation adding TCEP 10 mM and 2-Chloroacetamide 40 mM in urea buffer on the top of the filter and incubate for 30 min at 25°C. Specifically, add 200 μL of urea buffer to the filter plus 8 μL of 2-Chloroacetamide 1 M and 2 μL of TCEP 1 M. After 30 min, centrifuge 10 min at 9,300 × *g* at 25°C. Discard the flow through.
17. Exchange buffer by centrifugation at 9,300 × *g* for 10 min with 200 μL of buffer G (dilution buffer provided with the enzyme) and add 1.5 μL PNGase F (500 units/μl) for 1 h in 100 μL of buffer G at 25°C following manufacturer's instruction.

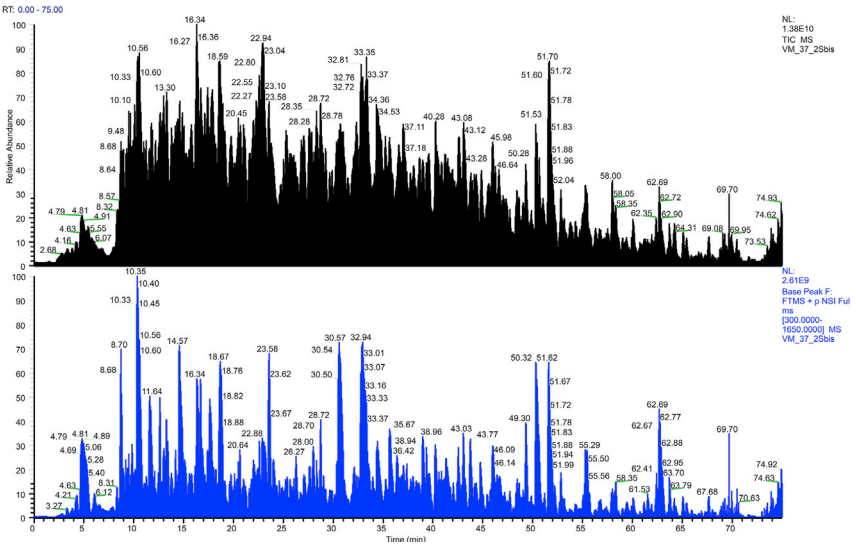

Figure 2. TIC (Upper Panel) and Base Peak Chromatogram (Lower Panel) of a Typical Secretome MS Analysis

18. Exchange again buffer by centrifugation at 9,300 × *g* for 10 min with 300 µL ammonium bicarbonate 50 mM for two times and digest with trypsin (1:50 = enzyme: secreted proteins) dissolved in 100 µL ammonium bicarbonate 50 mM for 12–18 h at 37°C.
19. Peptides are recovered at the bottom of the microcon filters by centrifugation at 9,300 × *g* for 10 min and by adding two consecutive washes of 50 µL of NaCl 0.5 M.
20. Exchange again buffer by centrifugation at 9,300 × *g* for 10 min with 300 µL phosphate buffer (pH 7.8) for two times and add Glu-C (1:50 = enzyme: secreted proteins) dissolved in 100 µL of phosphate buffer (pH 7.8) on the top of the filters to further digest polypeptides.
21. Peptides are recovered at the bottom of the microcon filters by centrifugation at 9,300 × *g* for 10 min.

Note: trypsin- and Glu-C-digested peptides are analyzed separately at the MS and pooled together in MaxQuant analysis.

Peptides Purification and LC-MS/MS (Day 4)

Timing: 2 days or more (depending on the number of samples to be analyzed)

This step describes how to purify peptides and LC-MS/MS analysis.

22. Eluted peptides are purified on a C18 StageTip (Rappsilber et al., 2007). 3 µg of digested sample are injected onto a quadrupole Orbitrap Q-Exactive HF mass spectrometer (Thermo Scientific).

Pause Point: The peptide samples can be stored at −20°C for a few weeks.

23. Peptides separation is achieved on a linear gradient from 95% solvent A (2% ACN, 0.1% formic acid) to 55% solvent B (80% ACN, 0.1% formic acid) over 75 min (Figure 2) and from 55% to 100% solvent B in 3 min at a constant flow rate of 0.25 µL/min on UHPLC Easy-nLC 1000 (Thermo Scientific) where the LC system is connected to a 23-cm fused-silica emitter of 75 µm inner diameter (New Objective, Inc. Woburn, MA, USA), packed in-house with ReproSil-Pur C18-AQ 1.9 µm beads (Dr Maisch Gmbh, Ammerbuch, Germany) using a high-pressure bomb loader (Proxeon, Odense, Denmark).

24. The mass spectrometer is operated in DDA mode: dynamic exclusion enabled (exclusion duration = 15 s), MS1 resolution = 70,000, MS1 automatic gain control target = 3×10^6, MS1 maximum fill time = 60 ms, MS2 resolution = 17,500, MS2 automatic gain control target = 1×10^5, MS2 maximum fill time = 60 ms, and MS2 normalized collision energy = 25. For each cycle, one full MS1 scan range = 300–1,650 m/z (Figure 2), was followed by 12 MS2 scans using an isolation window of 2.0 m/z.
25. MS analysis is performed as reported in Matafora et al., 2020. Raw MS files are processed with MaxQuant software, making use of the Andromeda search engine (Cox et al., 2011). MS/MS peak lists are searched against the UniProtKB complete proteome database depending on the samples origin. Searches are performed on both trypsin- and Glu-C-digested peptides analyzed together for each sample by selecting, as fixed modification, alkylation of cysteine by carbamidomethylation, while, as variable modifications, oxidation of methionine, N-terminal acetylation, and N-Deamination. For R and K amino acids, multiplicity is set to 2 and modifications including medium- heavy-labeled R and K are considered. Trypsin and Glu-C specificity are used with up to two missed cleavages allowed. Mass tolerance is set to 5 ppm and 10 ppm for parent and fragment ions, respectively. Andromeda generates a reverse decoy database used to calculate the False Discovery Rate (FDR), which is set to <0.01 for peptide spectrum matches (PSMs). For identification, a minimum of two peptides identified per protein is required, of which at least one has to be unique to the protein group. The minimum ratio count used for quantification is 2 (Troubleshooting 4).

Note: LC-MS/MS parameters and post data analysis may vary depending on the instruments and software available in each laboratory.

EXPECTED OUTCOMES

Generally, from 2×10^6 cells as starting material, the amount of secreted proteins recovered is about 30 μg. The amount of recovered proteins strongly depends on the cell type, i.e., proficient secretory cells or non-secretory cells.

With the Secret3D workflow, the MS analysis of the digested proteins usually leads to the identification of roughly 2,000 proteins. We have analyzed more than ten different cell lines and the number of proteins identified is comparable (Matafora et al., 2020).

Here we report as an example a list of the top 50 most abundant proteins identified by Secret3D in IGR37 melanoma metastatic cells (heavy-labeled) versus IGR39 primitive melanoma cells (medium labeled) (Table 1). Notably, both cell lines are labeled either medium or heavy, therefore the only source of light labeled proteins is the serum. MaxQuant provides the list of proteins identified and quantified in heavy and medium conditions, but also the light condition. By comparing H/L and M/L ratios contaminant proteins coming from serum can be monitored in both samples, while, analyzing the H/M ratios, the IGR37 and IGR39 secretome abundances can be directly compared. For each protein identified, the number of H/M ratio counts is the number of peptide spectrum matches for which heavy/medium pairs were detected; the higher is this number, the better is the quantitation. Moreover, intensity values give an estimation of proteins abundance in each sample, this intensity is the summed intensity of all peptide matches for each protein (modified or not unique peptides are not taken in account).

Proteins identified by Secret3D were verified as belonging to the extracellular space (Figure 3A) by mapping the identified proteins in the subcellular localization database Compartments (https://compartments.jensenlab.org/Search). Moreover, most of the identified proteins were also found in the human cancer secretome database (HCSD) (Feizi et al., 2015), attesting that Secret3D is

Table 1. Example of Quantitation and Analysis of the Top 50 Proteins Identified by Secret3D in IGR37 Cells (Heavy Labeled) and IGR39cells (Medium Labeled)

Protein ID	Protein Name	Gene Name	Intensity Ratio H/M	Count Ratio H/M	Intensity M	Intensity H
P02649	apolipoprotein E	APOE	2.80E+04	178	5.83E+09	6.45E+11
Q08380	galectin-3-binding protein	LGALS3BP	2.30E+00	53	2.22E+11	2.83E+11
P07093-2	glia-derived nexin	SERPINE2	2.30E+00	30	1.83E+11	1.91E+11
P11047	laminin subunit γ-1	LAMC1	1.10E+00	43	2.02E+11	1.38E+11
P08670	vimentin	VIM	1.10E+00	52	2.63E+11	1.22E+11
O43852	calumenin	CALU	1.60E+00	48	1.56E+11	1.21E+11
O60568	procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	PLOD3	3.00E+00	53	8.55E+10	1.16E+11
C9J2H1	inter-α-trypsin inhibitor heavy chain H5	ITIH5	9.00E+00	27	3.25E+10	1.12E+11
G3XAI2	laminin subunit β-1	LAMB1	1.20E+00	70	1.48E+11	9.18E+10
Q96RW7	hemicentin-1	HMCN1	1.70E+00	9	9.24E+10	9.12E+10
P10451-4	osteopontin	SPP1	7.20E+00	10	3.17E+10	9.08E+10
P07237	Protein disulfide-isomerase	P4HB	1.20E+00	78	1.55E+11	8.57E+10
A0A0A0MTC7	laminin subunit α-4	LAMA4	1.00E+00	19	1.50E+11	8.46E+10
P62805	histone H4	HIST1H4A	3.80E+01	23	3.27E+09	7.89E+10
P06733	α-enolase	ENO1	1.50E+00	50	1.42E+11	7.85E+10
P60709	actin, cytoplasmic 1	ACTB	1.10E+00	97	1.96E+11	7.85E+10
P24821	tenascin	TNC	7.60E-01	61	1.84E+11	7.65E+10
Q6UVK1	chondroitin sulfate proteoglycan 4	CSPG4	3.10E+00	67	4.17E+10	7.17E+10
P02787	serotransferrin	TF	1.40E+01	22	7.38E+09	7.16E+10
U3KQK0	histone H2B	HIST1H2BN	3.40E+01	17	3.16E+09	7.08E+10
A0A087WTA8	collagen α-2(I) chain	COL1A2	2.70E+01	9	7.01E+09	6.57E+10
P05067-11	amyloid β A4 protein	APP	1.60E+00	11	5.80E+10	6.44E+10
P11021	78 kDa glucose-regulated protein	HSPA5	9.40E-01	60	1.29E+11	6.03E+10
Q16610	extracellular matrix protein 1	ECM1	3.00E+00	10	3.19E+10	5.74E+10
P30101	protein disulfide-isomerase A3	PDIA3	1.00E+00	65	1.17E+11	5.70E+10
J3KPS3	fructose-bisphosphate aldolase A	ALDOA	1.20E+00	39	1.12E+11	5.58E+10
Q8IUX7	adipocyte enhancer-binding protein 1	AEBP1	3.70E+00	11	2.56E+10	5.55E+10
P00338	L-lactate dehydrogenase A chain	LDHA	8.20E-01	54	1.64E+11	5.45E+10
P07996	Thrombospondin-1	THBS1	1.70E-01	49	5.50E+11	5.09E+10
P98160	basement membrane-specific heparan sulfate proteoglycan	HSPG2	1.60E+00	12	5.51E+10	5.01E+10
D3DQH8	SPARC	SPARC	8.40E-01	16	1.15E+11	4.93E+10
P01023	α-2-macroglobulin	A2M	3.50E-01	88	2.69E+11	4.87E+10
P62258	14-3-3 protein epsilon	YWHAE	9.20E-01	28	1.19E+11	4.72E+10
A0A087X0S5	collagen α-1(VI) chain	COL6A1	3.40E-01	49	2.63E+11	4.38E+10

(*Continued on next page*)

Table 1. *Continued*

Protein ID	Protein Name	Gene Name	Intensity Ratio H/M	Count Ratio H/M	Intensity M	Intensity H
A0A0A6YY95	C-type lectin domain family 11 member A	CLEC11A	1.20E+02	5	1.30E+09	4.31E+10
P26038	moesin	MSN	1.10E+00	34	7.93E+10	4.29E+10
O94985	calsyntenin-1	CLSTN1	1.10E+00	10	6.47E+10	4.14E+10
Q16769	glutaminyl-peptide cyclotransferase	QPCT	1.30E+03	47	571420000	4.01E+10
K7EK07	Histone H3	H3F3B	7.30E+01	12	1.79E+09	3.92E+10
P18065	insulin-like growth factor-binding protein 2	IGFBP2	1.50E+00	12	5.21E+10	3.89E+10
P00558	phosphoglycerate kinase 1	PGK1	2.60E+00	30	3.54E+10	3.81E+10
P07900	heat shock protein HSP 90-alpha	HSP90AA1	9.40E-01	47	1.17E+11	3.76E+10
P11142	heat shock cognate 71 kDa protein	HSPA8	8.50E-01	56	9.86E+10	3.70E+10
P13667	protein disulfide-isomerase A4	PDIA4	1.10E+00	40	6.33E+10	3.65E+10
Q99988	growth/differentiation factor 15	GDF15	1.50E+03	63	1.58E+09	3.41E+10
O43707	α-actinin-4	ACTN4	5.30E-01	52	1.66E+11	3.41E+10
P07195	L-lactate dehydrogenase B chain	LDHB	9.70E-01	40	8.36E+10	3.29E+10
P02545-2	prelamin-A/C	LMNA	3.30E-01	61	1.95E+11	3.11E+10
Q96JB6	lysyl oxidase homolog 4	LOXL4	1.70E+00	4	2.75E+10	3.03E+10

suitable for secretome analysis (Figure 3B). Further, about one third of the secreted proteins identified were also predicted as secreted or transmembrane proteins in the Human Proteins Atlas database (www.proteinatlas.org) (Figure 3C) (Uhlén, et al. 2015).

Secreted3D is also able to provide information about the putative N-glycosylation sites of the identified secretome. Modified peptides are about 25%–30% of all identified peptides (Figure 4). As spontaneous deamination of asparagine can occur also without PNGase F participation, the glycosylated sites identified by N-deamidation in MS analysis need further validation (Palmisano et al. 2012).

Moreover, Secret3D leads to an increased protein sequence coverage compared to traditional methods, which do not include de-glycosylation and double digestion (Figure 5).

QUANTIFICATION AND STATISTICAL ANALYSIS

SILAC ratios from DDA .raw files are calculated by MaxQuant software. The statistical analysis is performed by using Perseus software included in the MaxQuant package. t test, Volcano plot, and ANOVA statistical analyses are performed depending on the experimental design. FDR <0.05 or p-value <0.05 is applied. KEGG enrichment pathway analysis is performed via EnrichR (http://amp.pharm.mssm.edu/Enrichr), using the Gene ID of the identified proteins.

As an example, we report here the Volcano plot of the secretome of metastatic (H) versus primitive (L) melanoma cell lines (Figure 6).

At least three biological replicates for each condition are required.

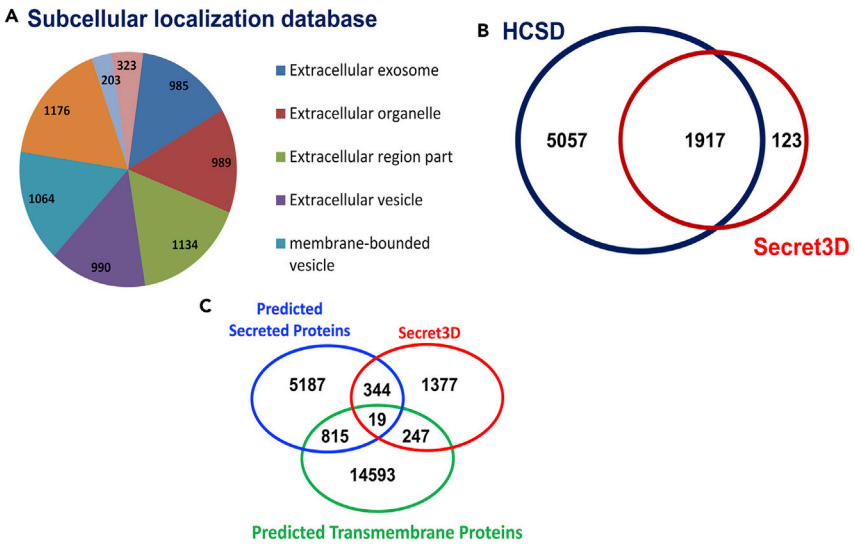

Figure 3. Subcellular Component Analysis of Secreted Proteins Identified by Secret3D Workflow

(A) Subcellular localization analysis performed by using the Compartments database.

(B) Venn diagram comparing the secreted proteins identified by Secret3D with the human cancer secretome database.

(C) Venn diagram comparing the secreted proteins identified by Secret3D and predicted secreted and transmembrane proteins in the Human Proteins Atlas Database.

LIMITATIONS

Secret3D protocol can be applied only to cell lines that are able to grow in conditioned medium. There are some cell lines that suffer when BSA is removed. Therefore, we recommend verifying cell viability upon conditioning. Viability has to be at least 95%. This protocol is not suited for tissues.

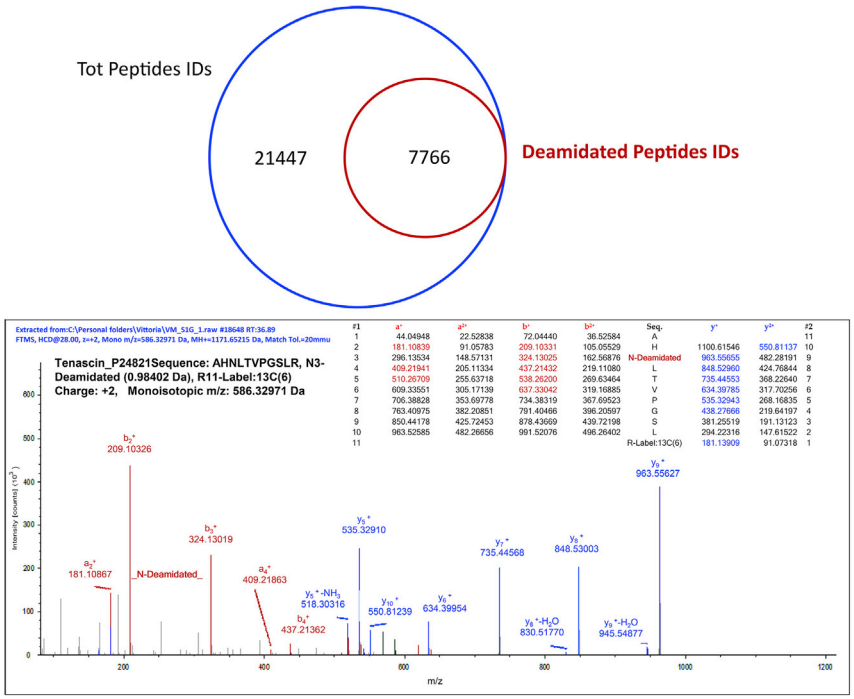

Figure 4. Analysis of Asn Deamidated Peptides in IGR37 versus IGR39 Cell Lines

Upper panel: Venn diagram indicating the modified peptides and total peptides identified. Lower panel: MS/MS spectrum of a tenascin glycosylated peptide.

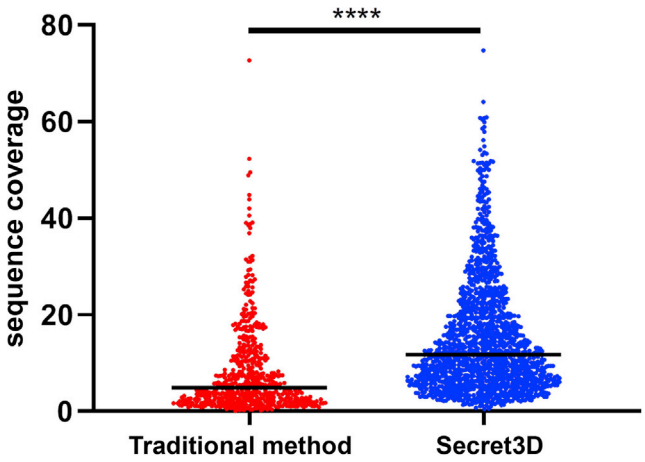

Figure 5. Sequence Coverage Analysis
Comparison of the sequence coverage of the proteins identified in IGR37 versus IGR39 cell lines by Secret3D or by a method without de-glycosylation and double digestion. t test: ****p < 0.00001.

This protocol is used to compare secretome from two different samples (medium versus heavy) but can modified for larger experiments including more than two conditions (see paragraph below).

TROUBLESHOOTING

Problem 1

Secretome analysis of more than two conditions or multiple cell lines (step 2).

Potential Solution

All the cells are heavy labeled, are run separately at the MS, and are treated in MaxQuant as for label free analysis (considering only heavy-labeled proteins).

Problem 2

Cell viability is under 95% (step 5).

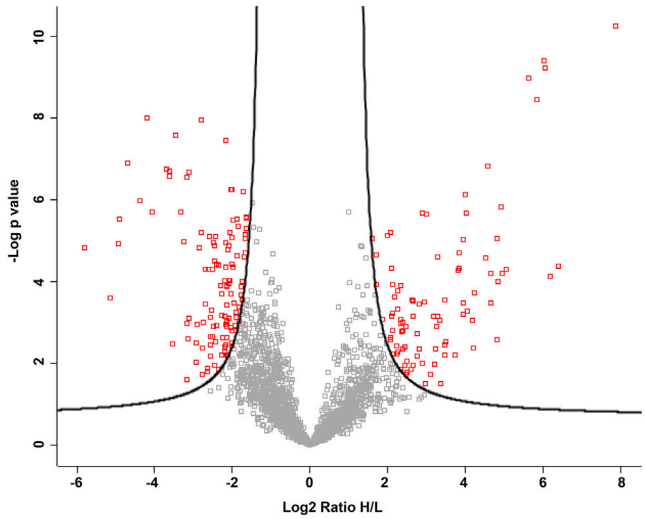

Figure 6. Example of Volcano Plot Representation of Significantly Modulated Proteins (Red Dots)
Intensity Ratio H/L of the proteins identified in metastatic (H) versus primitive (L) melanoma cell lines and p values are reported in the graph. The analysis was performed on three biological replicates. t test analysis, FDR <0.05, was used to select significant proteins.

Potential Solution

Reduce the conditioning time, 12 h instead of 18 h.

Problem 3

Cells secrete low amount of proteins (step 15).

Potential Solution

Start with more cells or increase time of conditioning, checking cell viability.

Problem 4

Secretome contains albumin contamination (step 25).

Potential Solution

Increase washing before cell starvation.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Angela Bachi (angela.bachi@ifom.eu).

Materials Availability

This study did not generate any unique materials or reagents.

Data and Code Availability

Proteomic datasets produced in this study are available in the following databases: Proteomics Identification database PeptideAtlas http://www.peptideatlas.org/, code PASS01358.

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AUTHOR CONTRIBUTIONS

V.M. was responsible for conducting the experiments and manuscript preparation. A.B. provided oversight and insight during the experiments and manuscript preparation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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