Electronic Supplementary Information (ESI)

Solid-phase synthesis of Rhodamine-110 fluorogenic substrates and their application in forensic analysis

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Reagents

All amino acids and peptide coupling reagents were purchased from Sigma Aldrich (Dorset, UK). Rhodamine-110 was obtained from Fisher Scientific Ltd (Loughborough, UK), whilst 2-Chlorotrityl Chloride Resin (1.45 mmol/g loading capacity) was purchased from Cambridge Bioscience (Cambridge, UK). All reagents were used without further modification, except DMF which was kept under constant nitrogen flow.

Resin-Rh-110-NH_2

Chlorotrityl resin (175 mg, 0.27 mmol) was stirred in DCM (1 mL) in the presence of N,N-Diisopropylethylamine (DIPEA, 700 µL, 4.11 mmol) for 10 min and subsequently added to round-bottom flask containing a solution of Rhodamine-110 (136 mg, 0.411 mmol) in DCM (5 mL) and DMF (2 mL). The mixture was gently agitated for 24 hrs before being transferred to an empty fritted polypropylene tube where the resin was filtered and washed with DMF (×5) and DCM (×10). Finally, the resin was incubated with methanol for 10 min to cap remaining chloride groups before being re-washed with DMF and DCM. Conjugation was monitored via a 'hard' cleavage of 5mg of resin to isolate Rhodamine-110 from the solid support via treatment with a 95:0.5:0.5:0.5:0.25 solution of trifluoroacetic acid (TFA): water: phenol: thioanisole (TA): triisopropylsilane (TIPS) for 3 hours at room temperature. Ice cold diethyl ether was then added to precipitate products, with organic solvent, scavengers and by-products removed by centrifugation. Obtained solids were washed several times with diethyl ether to remove any residual scavenger traces and finally dissolved in 50% acetonitrile (acn) in water containing 0.1% TFA and freeze-dried for 24 hrs Correct product mass was verified by ESI-MS on a Waters/Micromass (Manchester, UK) ZQ mass spectrometer (Fig. S1).

Resin-Rh-110-Gln(Trt)-OH

1.83 g (3 mmol) of Fmoc-Gln(Trt)-OH was added in a round-bottom flask to 8 ml of cold DMF/pyridine (1:1. v/v) and stirred in an ice bath until dissolved. To this was added 479 mg (2.5 mmol) of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC). After 5 min of stirring, 100 mg (0.1 mmol) of Rhodamine-110 resin, suspended in 2 ml of DMF/pyridine (1:1 v/v), was added. Gentle stirring was continued for 24 hrs at room temperature. The resin was transferred to an empty fritted polypropylene tube,filtered and washed with DMF (×5) and DCM (×10) before being re-incubated with additional Fmoc-Gln(Trt)-OH for another 24 h using the above-described procedure. Successful amino acid coupling was monitored by a 'soft' cleavage of 5mg of resin to isolate $_2$HN-Rh-110-Gln(Trt)-OH from the solid support whilst keeping acid-labile side-chain protecting groups intact. This was achieved by treating the resin with a 1ml solution of 1:1:8 acetic acid:TFE:DCM for 1 hour at

room temperature. Next, the resin was filtered off and washed with cleavage solution, with the collected filtrate concentrated under a stream of nitrogen at 40°C. Hexane (x15 volume excess) was added to precipitate the product, and subsequently removed by rotary evaporation. The obtained products were finally dissolved in 50% acn in water containing 0.1% TFA and freeze-dried for 24 hours. The correct mass of the products were once again confirmed via ESI-MS as already outlined (Fig. S2).

Resin-Rh-110-QLKSSH-Ac

Remaining amino acids were added to Rho110-Gln(Trt)-OH-conjugated resin via a standard Fmoc-Solid Phase Peptide Synthesis (SPPS) strategy. Initial Fmoc deprotection was accomplished by treatment with 20% (v/v) piperidine in DMF for 10 min. The following acylation steps were conducted using six equivalents of Fmoc amino acids, which were pre-activated with ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) and N,N'-Diisopropylcarbodiimide (DIPCDI) in DMF in a molar ratio of 1:1:1 of amino acid, Oxyma and DIPCDI, respectively. The N-terminal histidine was acetylated via the addition of ten equivalents of acetic anhydride and twenty equivalents of DIPEA in DMF. All coupling reactions were conducted for at least 1 hour. Excess reagents and impurities were removed by extensive washing with DMF, methanol and DCM. A 'soft' cleavage was once again performed using the above described method to remove $_2$HN-Rho110-QLKSSH-Ac from the solid support.

Ac-Rho110-QLKSSH-Ac

To $_2$HN-Rho110-QLKSSH-Ac (7mg) dissolved in DMF (1 ml) were added forty equivalents of DIPEA (28 µl) and twenty equivalents of acetyl chloride (5.7 µl). The resulting mixture was stirred for 48 h before the addition of 1 ml DCM and 1ml distilled water to isolate a final product by liquid-liquid extraction. The organic phase was isolated prior to the re-extraction of the aqueous layer with 1ml of fresh DCM. The organic layers were combined and washed with 5% $NaHCO_3$ (1ml) and brine (1ml) solutions and dried with sodium sulfate. Following filtration the solvent was removed via rotary evaporation. To remove acid-labile amino acid side-chain protecting groups, products were treated with using the same 'hard' cleavage cocktail solution and precipitation methods as previously outlined. Product purity was examined by analytical RP-HPLC (Fig. S3), carried out on a HP1050 HPLC system equipped with an autosampler, quaternary pump and Diode-Array Detector and employing a Zorbax SB C-18 2.1mm x 10cm (particle size 3.5 micron) column. A linear gradient of mobile phase B (acetonitrile containing 0.1% TFA) over mobile phase A (0.1% TFA in water) from 0-90% B in 20 minutes was performed with a flow rate of 0.2mL/min. Eluents were monitored at wavelengths between 210-280 nm with data collected and analyzed using ChemStation software. Correct product mass was determined by HRMS on an Exactive Plus Orbitrap Mass Spectrometer (Fig. S4).

Body Fluid Collection and Storage

Semen samples were taken after informed consent and stored at 4 °C until analysis. Tissue collection for this project has full ethical clearance under the King's College London Biomedical Sciences, Dentistry, Medicine and Natural & Mathematical Sciences Research Ethics Subcommittee (Reference number **BDM/13/14-26**).

Spectrofluorometry

Final substrate Ac-Rho110-QLKSSH-Ac (0.9 mg, 0.82 µmol) was dissolved in DMSO (82 µl) to make a 10 mM stock solution before dilution in 10 mM Tris reaction buffer to a 50 µM working concentration. Substrate performance was observed via the 100 µl addition of 50 µM Ac-Rho110-QLKSSH-Ac to 100 µl of both purified PSA protein (100, 50, 25, 12.5, 6.25 µg/ml) and seminal fluid dilutions (1:1, 1:2, 1:4, 1:8, 1:16) and measured with appropriate negative controls (100 µl 10 mM Tris Buffer, 100 µl assay reagent). Additional observations of substrate specificity were undertaken via the incubation of 50 µM Ac-Rho110-QLKSSH-Ac with 100 µg/ml concentrations of Proteinase K (Fisher Scientific Ltd, Loughborough, UK), Aminopeptidase M (Merck Millipore, Darmstadt, Germany) and Trypsin (Sigma Aldrich, Dorset, UK) (Fig. S5). All fluorescence measurements were conducted on a BioTek Synergy HT spectrophotometer (Vermont, USA). Emissions were recorded at room temperature in triplicate using Ex485±20/Em528±20 nm wavelengths (for the measurement of emissions at 535 nm) over the course of 30 minutes.

***In Situ* Microscopy**

Six different surfaces consisting of cotton, glass, leather, paper, plastic and wood were chosen to reflect materials on which body fluids are commonly deposited within criminal investigations. All surfaces were cut to fit the size of a microscope slide. Semen volumes of 5 µl were applied to the centre of each surface before the direct 5 µl addition of substrate Ac-Rho110-QLKSSH-Ac. Negative reagent-only and blank semen-only controls were used to limit the possibility of background fluorescence. Images were taken in the dark immediately after application on an Olympus SZX12 fluorescence microscope. GFP filtration (Ex 460-490 nm) was used for substrate excitation, whilst all additional microscopy parameters were kept constant (Hue 359, Saturation 255, White Balance 0, Contrast 1023, Brightness 0, Gamma 10, Magnification X8.5) in order to restrict result variation.

***In Situ* Specificity**

An additional observation of substrate specificity against other body fluid types was undertaken via the incubation of 5 µl substrate Ac-Rho110-QLKSSH-Ac with 5 µl of blood and 5 µl saliva deposited on glass slides. Controls and fluorescence microscopy were utilised in the same manner as previously described.

DNA Profiling

5 µl of Ac-Rho110-QLKSSH-Ac at working concentration was added to 5 µl of human semen deposited on glass slides to observe effects on DNA recovery after application. The resulting mixture was then recovered via the use of a cotton swab. DNA was extracted using the QIAmp® DNA Mini kit (Qiagen, Manchester, UK) according to the supplied protocol and quantified with the Quantifiler® Human DNA Quantification Kit (Fisher Scientific Ltd, Loughborough, UK). Samples were diluted to 0.5 ng/µl prior to amplification with the PowerPlex® ESI 17 Pro System (Promega, Southampton, UK) using a Perkin Elmer 9700 thermal cycler (Cambridge, UK). STR amplicons were resolved on an ABI3130XL genetic analyser and evaluated using GeneMapper® software. Generated profiles were compared to a semen reference profile (5 µl semen + 5 µl H_2O to examine potential inhibition (Fig.S7).

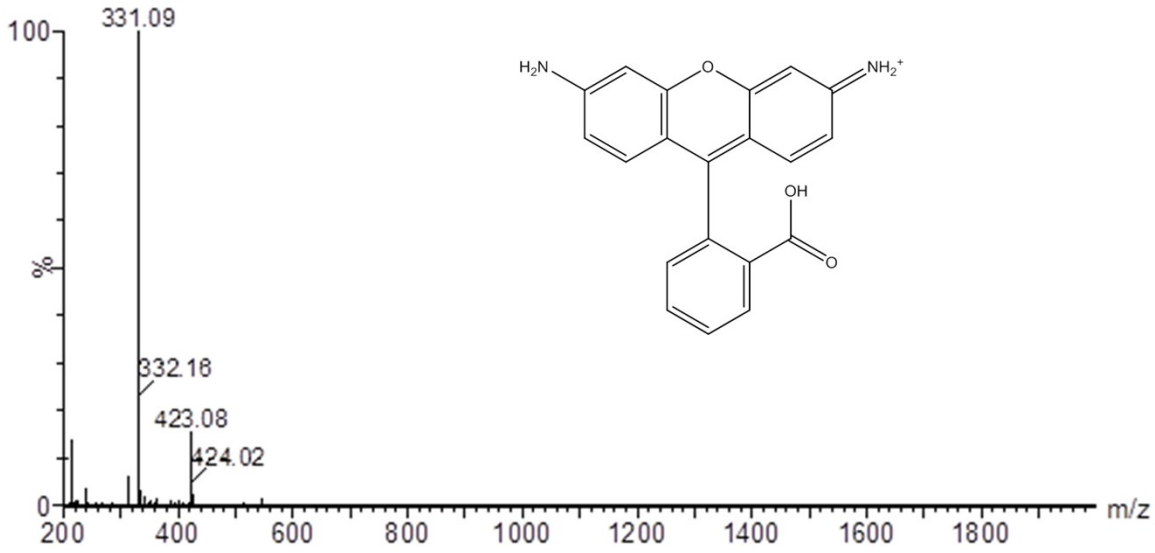


Fig. S1 ESI-MS data demonstrating free Rhodamine-110 presence after cleavage from a solid support (calculated m/z for $[M+H]^+$ = 331.11).

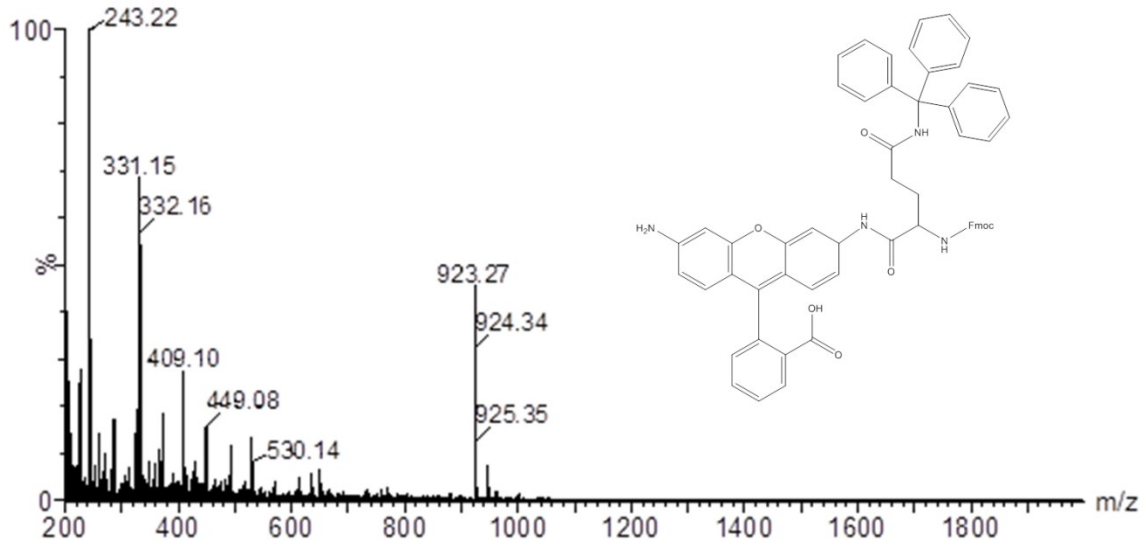


Fig. S2 ESI-MS data demonstrating successful Resin-Rhodamine-110-Glutamine conjugation after 'soft' cleavage from a solid support (calculated m/z for $[M+H]^+$ = 923.34).

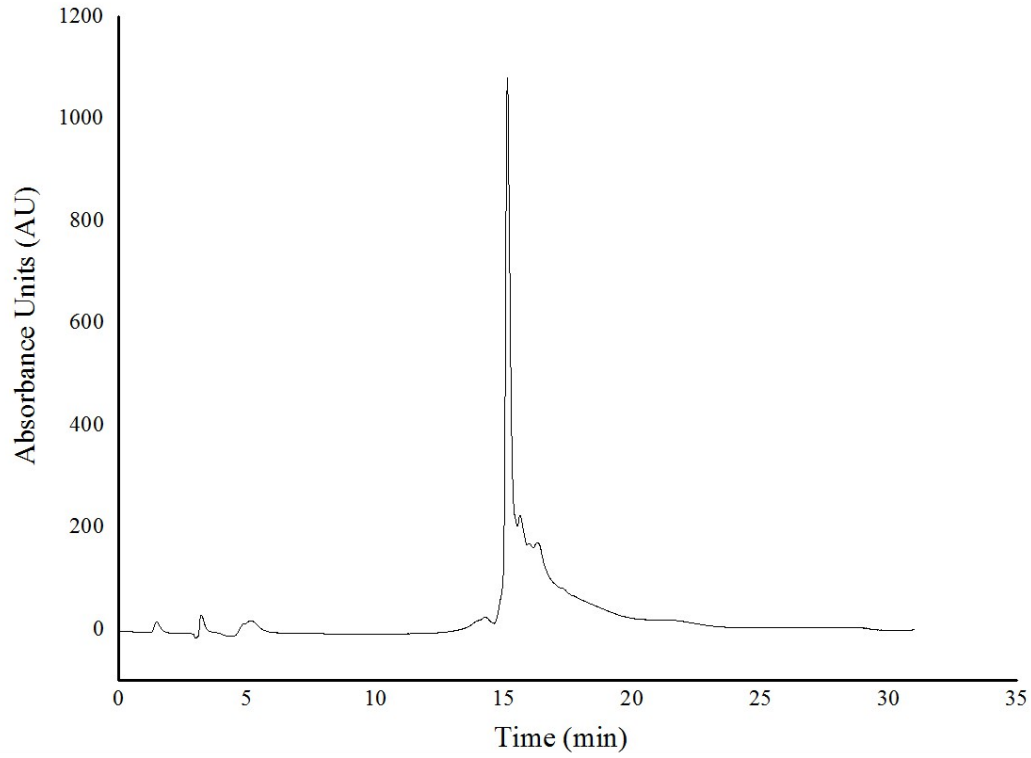


Fig. S3 RP-HPLC data to demonstrate purity of final substrate product Ac-Rh-110-QLKSSH-Ac

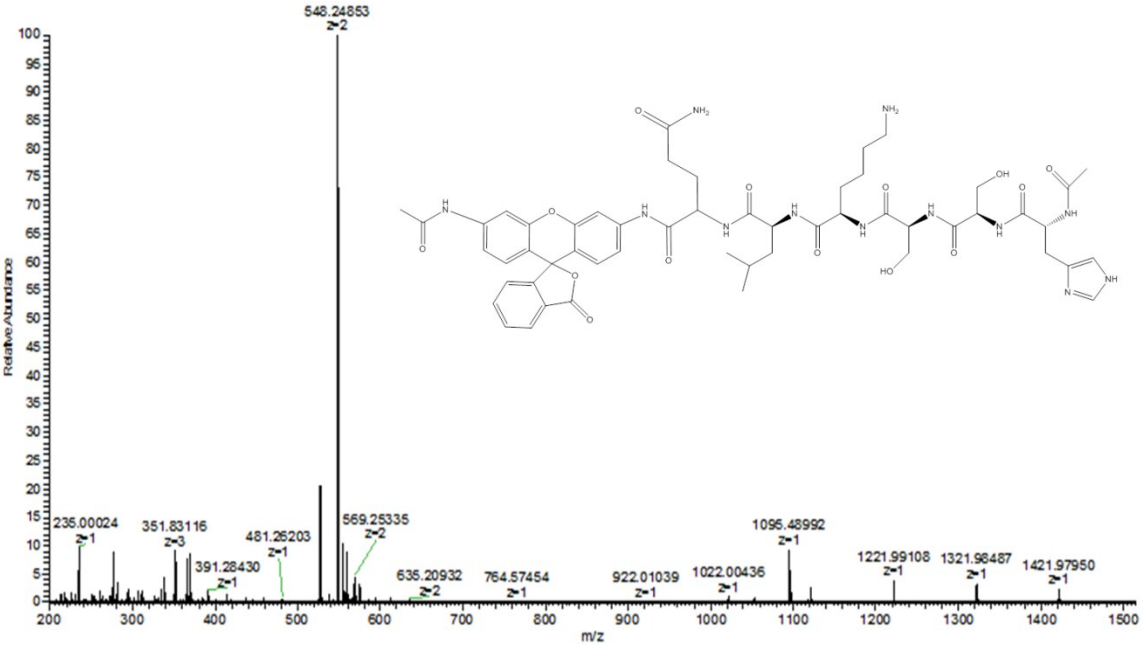


Fig. S4 High-resolution MS data of final substrate product Ac-Rh-110-QLKSSH-Ac (calculated m/z for $[M+H]^+$ = 1095.4900) and doubly charged $[M + 2H]^{2+}$ ion (calculated m/z = 548.2489)

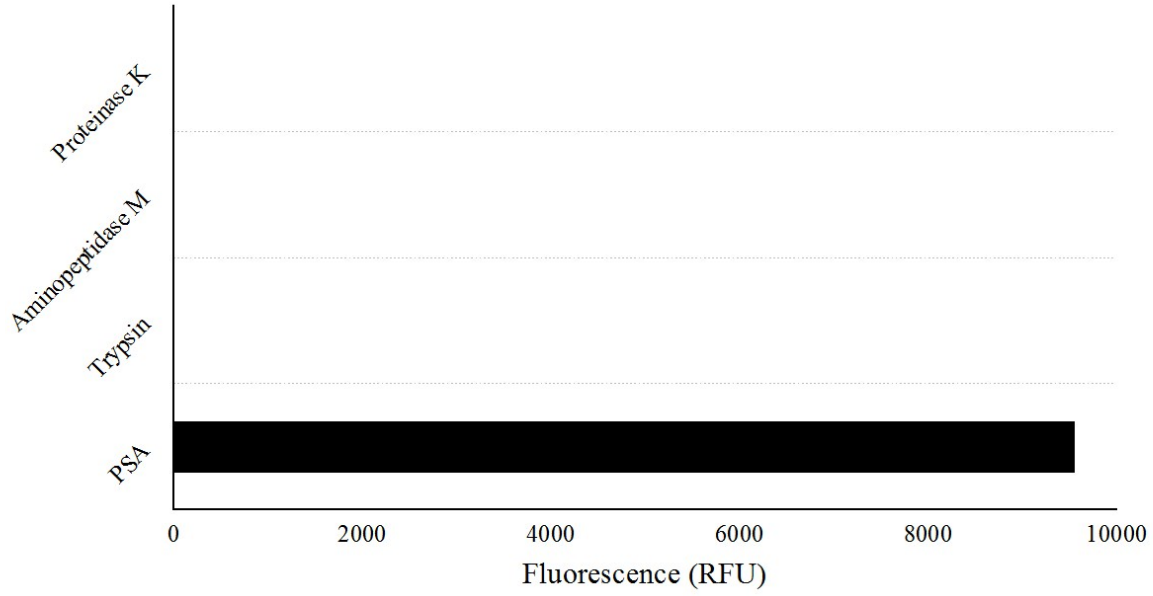


Fig. S5 Fluorescence response of Ac-Rh-110-QLKSSH-Ac to 100 µg/ml concentrations of Proteinase K, Aminopeptidase M, Trypsin and PSA.

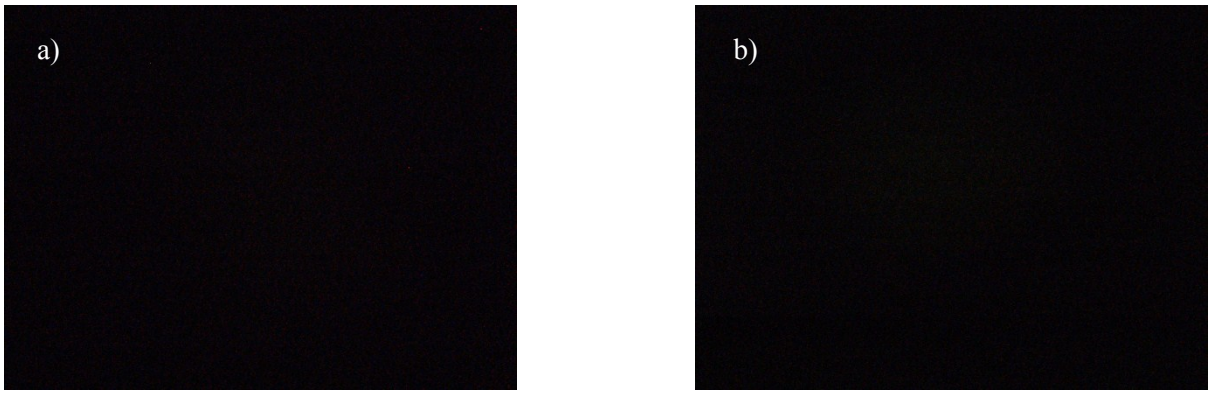
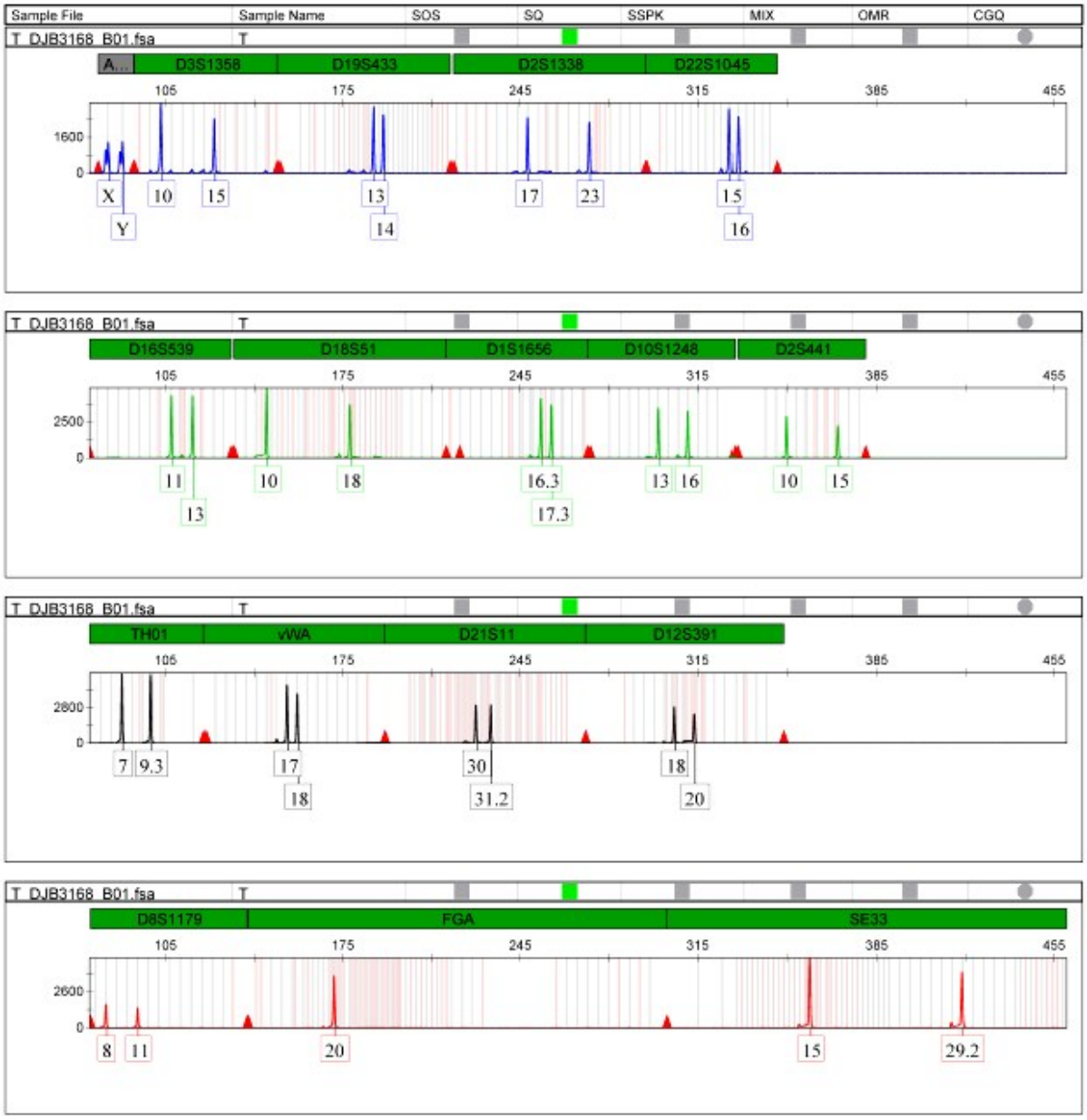


Fig. S6 Incubation of substrate Ac-Rh-110-QLKSSH-Ac with a) blood and b) saliva. No positive signals were observed upon application.

a)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b)

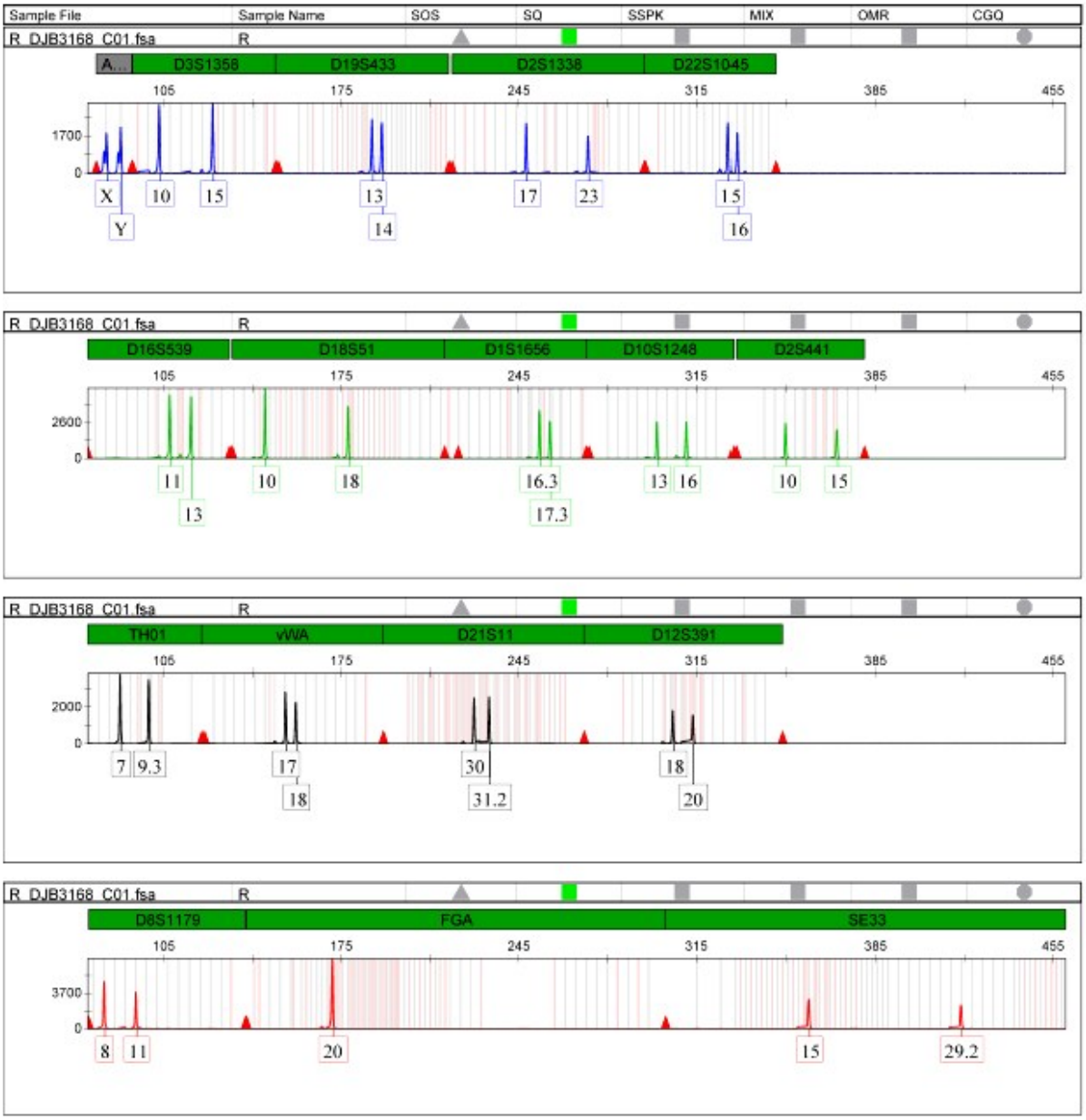

Fig. S7 Generated STR profiles from a) human semen sample treated with 5 µl substrate Ac-Rho110-QLKSSH-Ac and (b) reference human semen sample treated with 5 µl H_2O. No significant differences between profiles was observed.