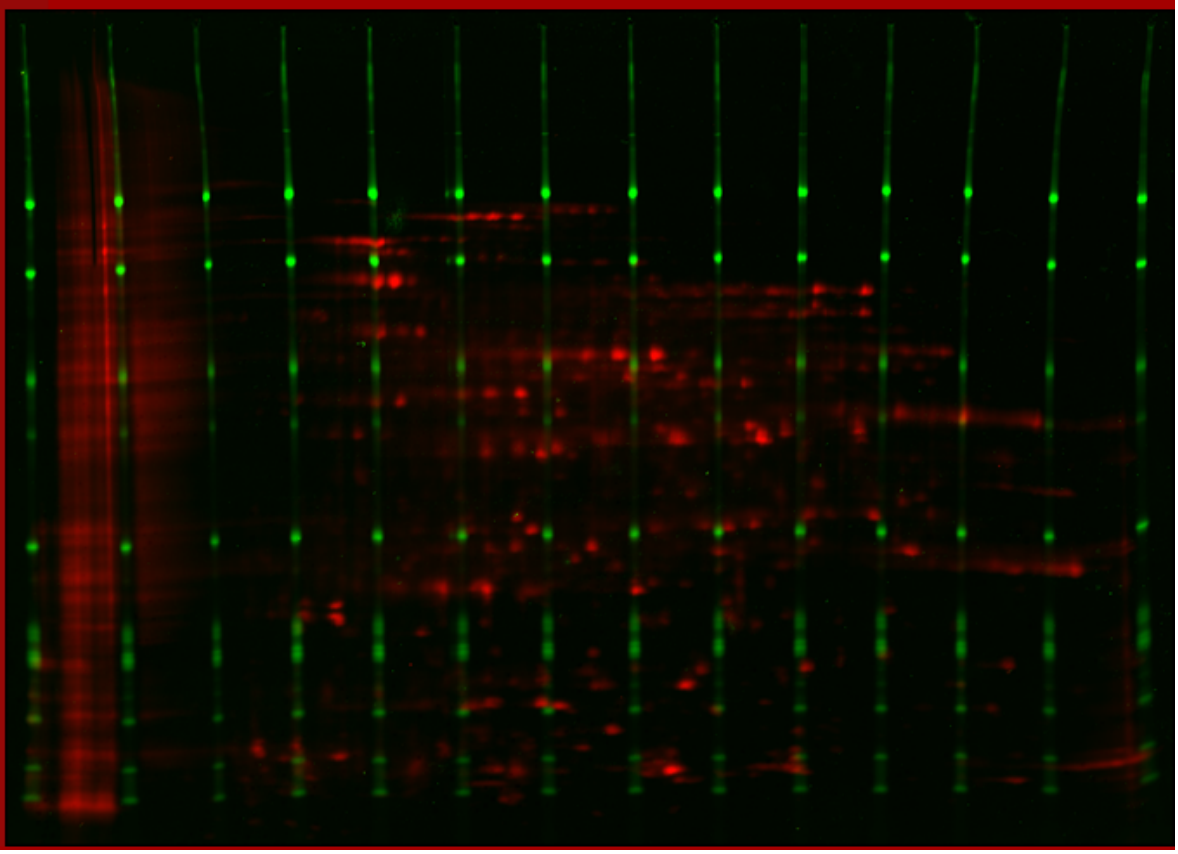
Volume 1 · Number 2 · October 2017

MERCATOR Journal Biomolecular Analysis

Laboratory Protocols

Mass spectrometry & Proteomics

Editor Simone König



MERCATOR Journal Biomolecular Analysis Vol 1 · No 2 · Oct. 2017 · pp 1-11

Digital laboratory notes of the Core Unit Proteomics CUP
Interdisciplinary Center for Clinical Research IZKF
Medical Faculty
Westfalian Wilhelms University of Münster
Germany
https://campus.uni-muenster.de/cu-proteomics

Edited by Simone König
CUP/IZKF, Röntgenstr. 21, 48149 Münster
koenigs@uni-muenster.de
Ph. +49-251-8357164

Cover image
Mercator gel (run by D. Ackermann at CUP) representing the award-winning CoFGE technology for standardized gel electrophoresis

Protocol

Bradykinin assay for monitoring serum protease activity

*Malte Bayer, Simone König**

Core Unit Proteomics, Interdisciplinary Center for Clinical Research, University of Münster, Germany

*Corresponding author: koenigs@uni-muenster.de

Abstract

A step-by-step protocol for monitoring serum protease activity using dabsylated bradykinin is provided. Detection is performed using thin-layer chromatography. The assay can be run with as little as 3 µl of serum and responds particularly well in cases when angiotensin-converting enzyme activity is changed.

Introduction

In the context of Complex Regional Pain Syndrome (CRPS) [1, 2] a thin-layer chromatography (TLC)-based method was developed to monitor serum protease activity [3]. This assay has proven particularly useful to measure the activity of angiotensin-converting enzyme (ACE) when using bradykinin as a substrate as it has been indicated that ACE activity is lowered in CRPS [1]. Below, a step-by-step protocol is given to (1) label bradykinin (BK) with dabsyl chloride to generate a coloured product (DBK), (2) purify the product, (3) perform the peptide assay, and (4) analyse the data.

Material

Equipment

1. Vortex mixer
2. Speedvac concentrator (ThermoScientific)

for dabsylation of bradykinin

3. Omix tips or equivalent C18 phase based SPE (100 µL, Agilent)
4. Äcta Start (GE Healthcare) with cation exchange column (Hi Trap SP FF) or equivalent chromatographic system

for TLC-based assay

5. Thermomixer
6. Cooling rack / thermoblock
7. Benchtop centrifuge
8. Centrifuge with cooling capabilities
9. Flatbed scanner
10. Gelloader pipette tips (Millipore)
11. Conventional hair dryer
12. TLC-chamber (for use with 10 cm x 10cm TLC plates, Sigma-Aldrich, Z266019)

Buffers, solutions and chemicals

1. Methanol (MeOH)
2. H_2O_{chr}

for preparation of dabsylated bradykinin

3. BK (Sigma), in water
4. 0.4 M $NaHCO_3$, pH 8.6
5. Acetonitrile (ACN)
6. Dabsyl chloride
7. Buffer A for cationic exchange: 10 mM TRIS pH 9.7 in 50 % ethanol (EtOH)
8. Buffer B for cationic exchange: 10 mM TRIS pH 9.7 in 50 % EtOH, 1M NaCl

for TLC based assay

9. Dabsylated BK (DBK), see preparation below; 527.2 pmol per sample
10. Acetone, precooled to -20°C
11. Acetic acid (HAc)
12. Chloroform
13. TLC silica gel 60 W, 20 x 20 cm (Merck)

Methods
Preparation of DBK

Dabsylation

1. Add BK (100 nmol in H_2O) to an 1.5 mL Eppendorf tube and in a speedvac
2. Add 5 µL $NaHCO_3$-buffer (0.4 M, pH 8.6) and 200 nmol dabsyl chloride (in 40 µL ACN, 5 mM)
3. Incubate at room temperature for 24 h
4. Dry sample in a speedvac

Clean-up procedure A (cation exchange liquid chromatography)

To improve recovery, dried samples can be resuspended in 100 µL MeOH and centrifuged (10 min, 20000 rpm) to remove most of the salts that are a side product of the reaction prior to cationic exchange. The supernatant is transferred to a new sample tube and the pellet is washed with 100 µL MeOH and centrifuged (10 min, 20000 rpm). Supernatants are combined and solvents are evaporated to dryness.

1. Resuspend dried reaction product in 1 mL buffer A
2. Run method outlined in Box 1
3. Combine all coloured (orange/yellow) fractions containing product
4. Dry samples and proceed with cleanup-procedure part 2

Box 1: LC-method for cationic exchange

1. Equilibrate system for 5 min (flow rate: 1 ml/min; 100% buffer A)
2. Sample injection: 2 min, 1 ml/min. As sample is solubilized in 1 mL buffer A, this method will wash the sample tube to maximize recovery
3. Elute unbound material (3 min, 1 mL/min, 100 % buffer A)
4. Collect 1 min-fractions, linear gradient from 100 % to 50 % buffer A in 15 min; 100 % buffer B for 5 min to to remove residual bound molecules, 1 mL/min

Clean-up procedure B (solid-phase extraction)

Smaller amounts of dabsylated peptides can be purified by C18-based solid phase extraction (SPE). To completely remove dabsyl chloride and side products SPE has to be repeated.

1. Resuspend crude reaction product in 50% MeOH
2. Wash and equilibrate Omix Tip (or equivalent) 3 times with 100 µl MeOH followed by 3 times 100 µl H_2O
3. Bind reaction product to C18 phase by pipetting up and down 30 times
4. Wash by pipetting and discarding 100 µl H_2O 3 times
5. Elute bound educts by fractionation into different sample tubes using a rising methanol concentration from 10% to 50% (10%-steps; two tubes for each concentration to ensure

equilibration at the desired concentration as some liquid of the previous solution remains in the tip). Fractions may be used to recover unreacted BK. As dabsyl chloride and dabsyl hydroxide is not separated completely by this technique, fractionation prior to eluting the product maximizes purity
6. Elute product to a fresh sample tube with 100 µl MeOH
7. To maximize yield, repeat steps 3-6 until no more product remains in the crude reaction (by yellow/orange colour of the C18 phase, i.e. C18 phase is colorless after step 5)
8. Evaporate product to dryness and proceed to clean-up procedure part 2

The fractionation step of the clean-up procedure should be optimized in case of different peptides. Here, the authors obtained relatively pure DBK (i.e. no educts could be detected by targeted MS/MS) by using this method (clean-up procedure B + clean-up procedure part 2).

Clean-up procedure part 2

In order to remove remaining unreacted BK after cation exchange clean-up and to further purify DBK from SPE another C18-SPE step is required.

1. Resuspend precleaned product in 50% MeOH and 50% ammonium bicarbonate buffer (50 mM pH 8.6)
2. Wash and equilibrate Omix tip 3 times with 10 µl MeOH followed by 3 times 10 µl H_2O
3. Bind reaction product to C18 phase by pipetting up and down 30 times
4. Wash by pipetting and discarding 10 µl H_2O 3 times
5. Separate educts and side products from product by eluting them to a series of 5 tubes containing 50% MeOH (100 µL) by slowly pipetting up and down 25 times. As most of the dabsyl chloride and its side products have been removed in the previous clean-up step, the solutions should remain mostly colorless
6. Purified reaction product is eluted to a sample tube containing 100 µL MeOH by pipetting up and down 30 times
7. Repeat steps 3 to 6 until C18 phase remains colorless after pipetting starting solution 30 times to maximize recovery
8. Concentration of DBK is measured via external calibration (mass spectrometry of BK)
9. Product is aliquoted to 500 µL Eppendorf tubes (527,2 pmol per tube) and evaporated to dryness
10. Aliquots can either be used directly or stored for at least 6 months in the dark at -20°C

Peptide Assay

Preparation of TLC chamber

1. Add 11 mL chloroform, 4 mL MeOH, 0.6 mL H_2O and 90 µL CH_3COOH to the TLC chamber and mix by gentle shaking
2. Before running samples, let the chamber saturate for at least 30 min
3. The TLC chamber can be cleaned by washing with MeOH and air-drying dry under a fume hood

Incubation of DBK with blood serum

1. For each sample to be analyzed place one Eppendorf tube with dried DBK (527,2 pmol) in a cooling rack (-20°C)
2. Pipette 3 µl of serum to the inner edge of the tube
3. Briefly centrifuge the sample tubes, vortex and centrifuge again
4. Properly cool samples during handling to minimize preliminary enzymatic digestion of DBK
5. Place all samples in a thermomixer preheated to 37°C and start timer
6. After the desired incubation period add 18 µL precooled acetone (-20°C) to the samples and freeze them for at least two hours (-20°C)
7. Centrifuge samples for 1 h (4°C, 20000 g) and pipette supernatant to new tube (Figure 1)

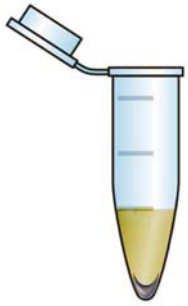


Figure 1: Precipitation of macromolecular material using ice-cold acetone. DBK fragments are in the supernatant.

8. Wash pellet by adding 20 µL acetone (-20°C), briefly vortex and centrifuge for 30 min (4°C, 20000 g)
9. Pool supernatants and dry in a speedvac

TLC

1. Prepare TLC sheet by marking starting points for each sample 2 cm above the lower edge
2. Add 1 µL MeOH to each sample, briefly centrifuge, vortex and centrifuge again to collect solution at the bottom of the vial
3. Place gelloader tip into sample tube. The sample solution will rise into the tip by capillary action within a few seconds
4. Spot sample solution by briefly touching a previously marked spot with the end of the gelloader tip multiple times. Avoid large sample spots (briefly let MeOH evaporate, Figure 2)
5. When the gelloader tip is empty, repeat steps 2-4 to improve recovery
6. Once all samples are spotted, dry all spots using a hair drier
7. Carefully place the TLC plate in the TLC chamber so that the mobile phase does not yet touch the spots (Figure 3)
8. Remove the TLC plate when mobile phase has almost reached the upper edge (1 cm distance)
9. Dry plate with hair drier and immediately scan it with a flatbed scanner
10. Store scanned TLC-plates in a cool, dry and dark place. Spots are still visible after 6 months and may be reexamined

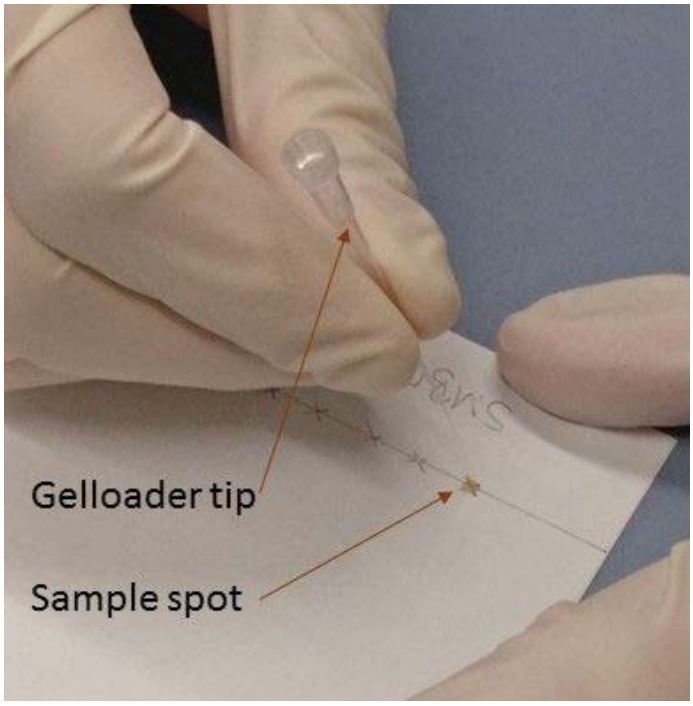
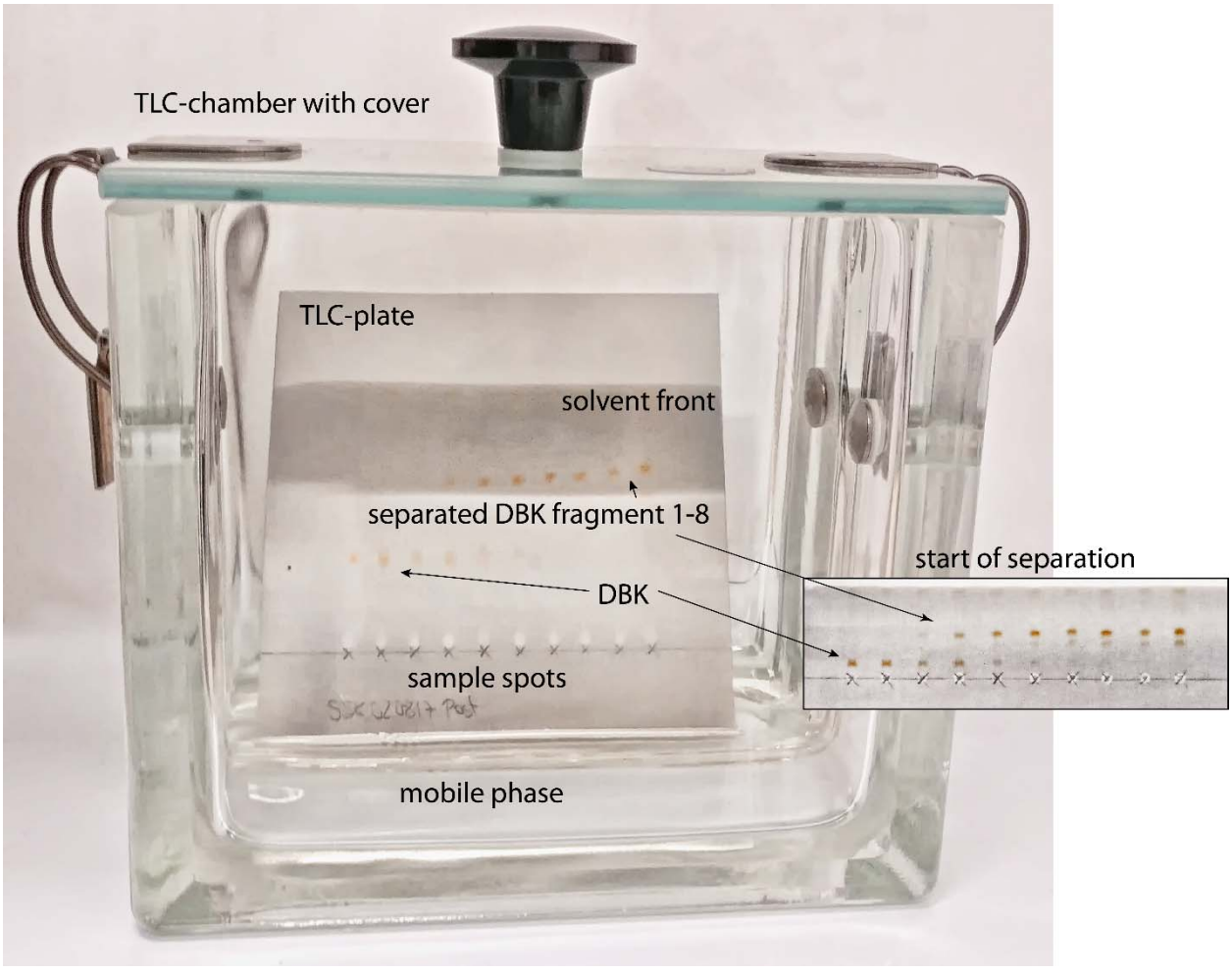


Figure 2: Sample spotting on pre-marked TLC-sheets using capillary action from gelloader tips.

Figure 3: TLC-plate during separation. The mobile phase runs through the plate by capillary action separating DBK from its fragments.



Data analysis

Scanning of TLC plates

1. As dabsylated peptides are sensitive to light, TLC plates have to be scanned immediately after the run is completed
2. The resolution should be set to a moderately high setting and automatic enhancements should be disabled to allow for a better plate-to-plate comparison
3. Contrast and color noise can be optionally removed using the Photoshop plug-in Dfine 2 (Google) in standard settings (Figure 4)

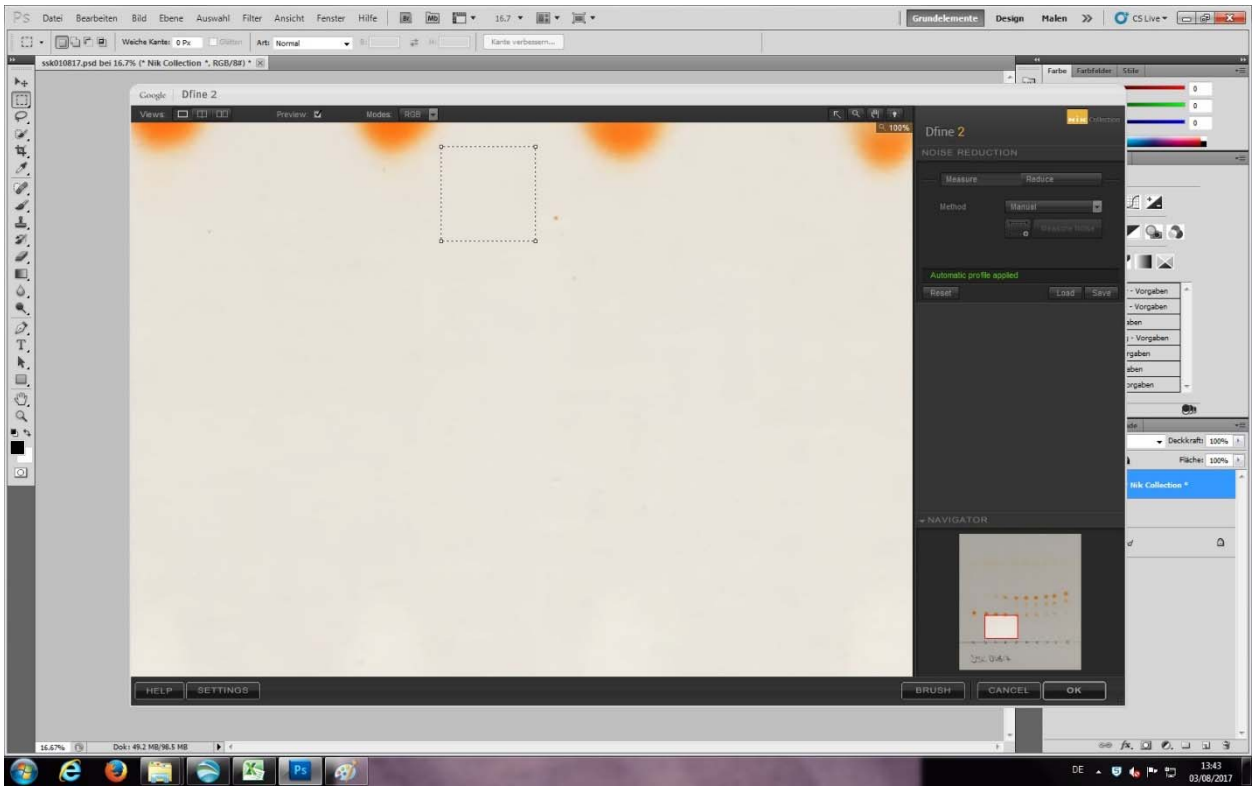


Figure 4: Use Dfine 2 (Google) to denoise scanned image.

5. By converting the images to black and white using Silver Efex Pro 2 (Google) with neutral settings and a blue filter, the local contrast can be improved significantly (Figure 5)
6. Images have to be saved as single layer tif to ensure compatibility with Quantity One software

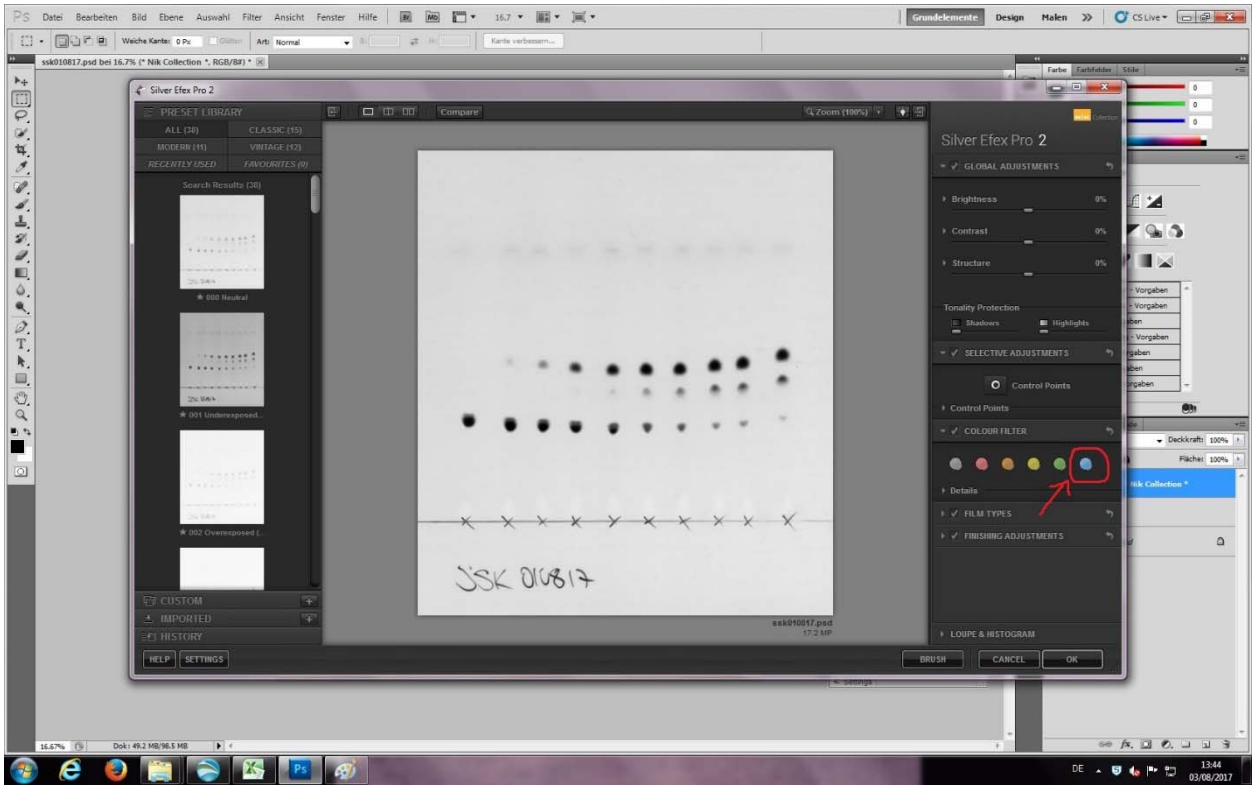


Figure 5: Use Silver Efex Pro 2 to convert image to black and white using neutral settings and blue filter (marked in red)

Image analysis with Quantity One software

1. Load modified image to Quantity One (GE) and define lanes for each sample on the TLC plate (Figure 6)
2. Remove lane background by applying rolling disk calculation and a disk size of 500 (Figure 7)
3. Mark all spots with the band tool. Depending on spot size, the value for band size may have to be adjusted (Figure 8)
4. Use band attribute “trace quantity” to get a readout for all marked spots (Figure 9)

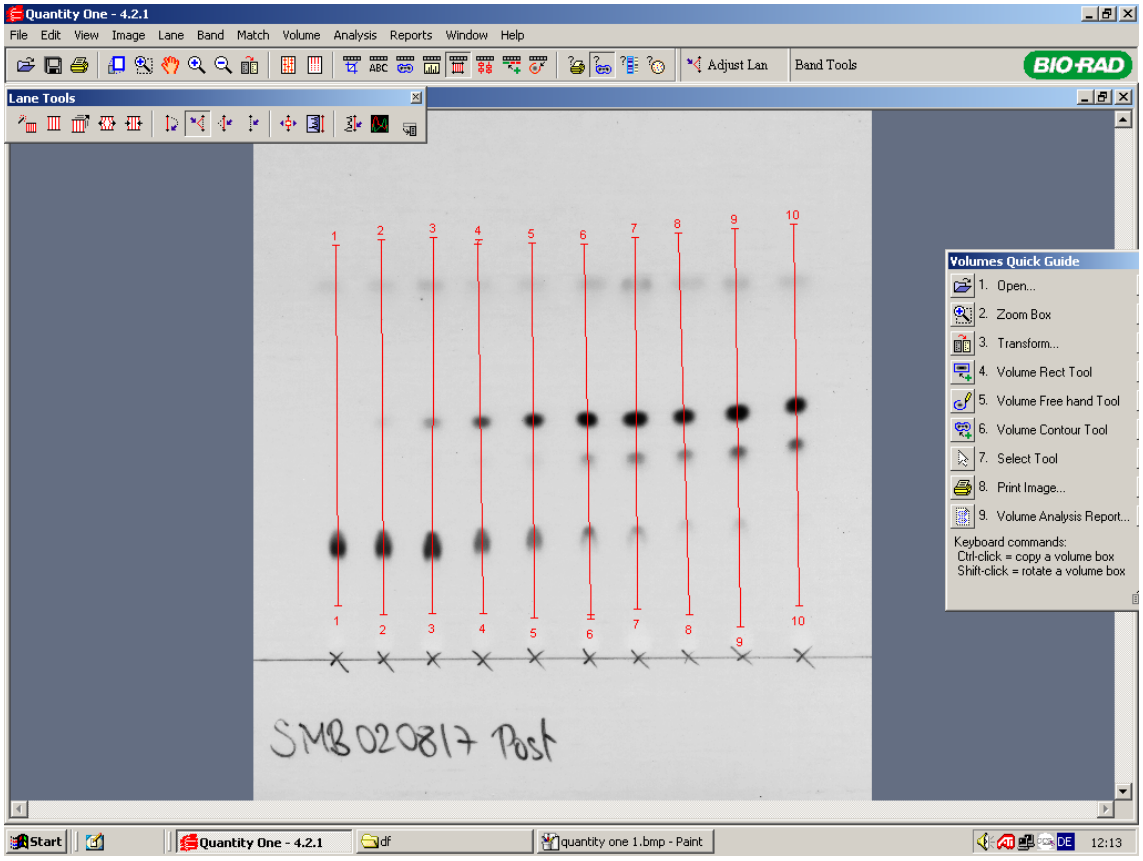


Figure 6: Mark all lanes on the TLC plate using the lane tool in Quantity One

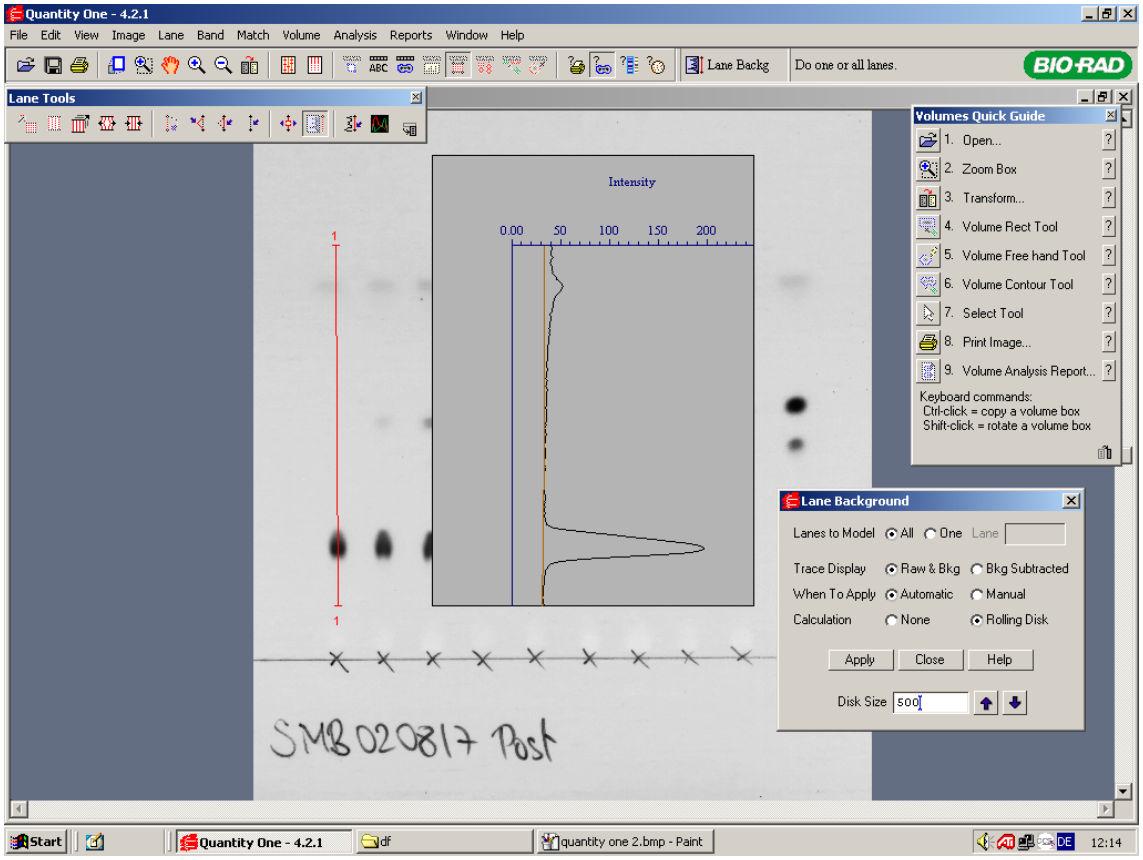


Figure 7: Remove lane background using rolling disk calculation (disk size 500)

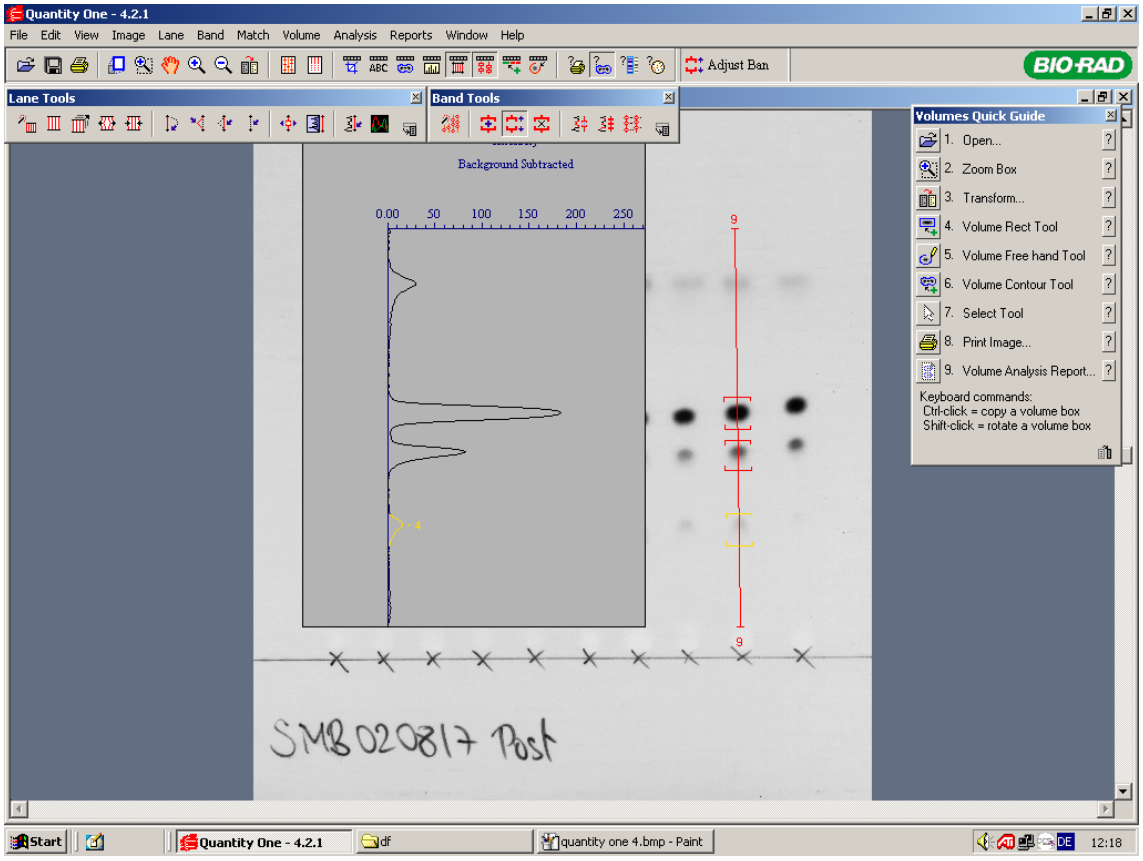


Figure 8: Mark all spots using Quantity One`s band tools

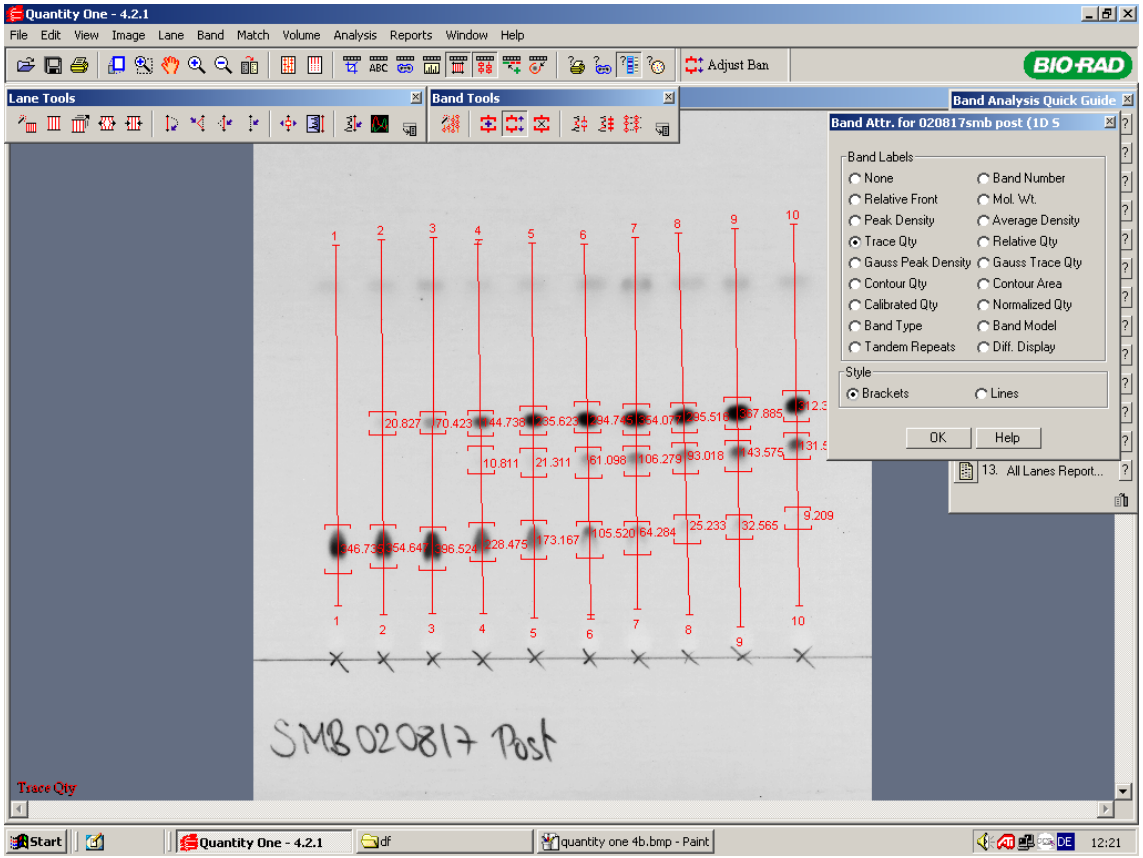


Figure 9: Use Trace Quantity to get a readout of the densities of all spots

Calculation

For further analysis, a number of parameters can be extracted from the data. The raw data, the density values per band (ρ), may be considered in a first step. In order to minimize volume errors and to allow for a better comparison among different time points and TLC plates, calculate the relative densities of each spot (density p of a selected spot divided by the sum of densities of all spots at this time point) e.g.:

$$r\rho_{(DBK1\text{-}5)} = \rho_{(DBK1\text{-}5)} / (\rho_{(DBK1\text{-}5)} + \rho_{(DBK1\text{-}8)} + \rho_{(DBK1\text{-}9)})$$

This calculation is useful when the impact of different enzymes is investigated. Different TLC plates and samples may also be compared using the (relative) densities of all spots after an incubation period of 60 min (plateau value) using the following formula:

$$\frac{\rho(\mathrm{DBK1-8})}{\rho(\mathrm{DBK1-5})} x (1 - \rho(\mathrm{DBK1-9}))^2$$

In this way, information about the overall change of protease activity with respect to certain sample sets (disease vs. control) can be gained. Alternatively, the slope of $\rho_{(DBK1\text{-}5)}$ versus time as well as the mean value for the height of the plateau of $\rho_{(DBK1\text{-}8)}$ occurring between 30 and 60 min may be used to compare samples.

Conclusion

Above protocol allows to monitor serum protease activity in a low-cost assay using dabsylated bradykinin with TLC separation. Only a few microliter of serum are required. Color density values of DBK fragments can be used to investigate if (1) serum protease activity differs between sample groups and if (2) ACE shows a higher or lower activity in the chosen context as BK is a substrate of ACE.

Acknowledgements

This work was supported by the Transfer Prize 2013/2014 of the University of Münster and DFG project KO1694/13-1

References

[1] König S, Schlereth T, Birklein F, 2017, Molecular signature for complex regional pain syndrome and its analysis. Expert Reviews in Proteomics 14:857-867.

[2] Schildt A, Schlereth T, Birklein F, König S, 2017, Analyzing marker substances for CRPS. Mercator J Biomol Anal 1 (2017) 1-23, http://nbn-resolving.de/urn:nbn:de:hbz:6-22249450016.

[3] Bayer M, König S, 2017, A vote for robustness: Monitoring serum enzyme activity by thin-layer chromatography of dabsylated bradykinin products. J Pharmaceut Biomed Anal 143:199-203.