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ORIGINAL ARTICLE

Dealing with Large Sample Sizes: Comparison of a New One Spot Dot Blot Method to Western Blot

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SUMMARY

Background: Western blot is the gold standard method to determine individual protein expression levels. However, western blot is technically difficult to perform in large sample sizes because it is a time consuming and labor intensive process. Dot blot is often used instead when dealing with large sample sizes, but the main disadvantage of the existing dot blot techniques, is the absence of signal normalization to a housekeeping protein.

Methods: In this study we established a one dot two development signals (ODTDS) dot blot method employing two different signal development systems. The first signal from the protein of interest was detected by horseradish peroxidase (HRP). The second signal, detecting the housekeeping protein, was obtained by using alkaline phosphatase (AP).

Results: Inter-assay results variations within ODTDS dot blot and western blot and intra-assay variations between both methods were low (1.04 - 5.71%) as assessed by coefficient of variation.

Conclusions: ODTDS dot blot technique can be used instead of western blot when dealing with large sample sizes without a reduction in results accuracy.

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KEY WORDS

one dot two development signals (ODTDS) dot blot, western blot, protein quantification, large sample size studies, comparison

INTRODUCTION

Western blot is the gold standard method to determine individual protein expression levels. However, western blot is technically difficult to perform in large sample sizes because it is a time consuming and labor intensive process. Dot blot is often used instead when dealing with large sample sizes [1-5]. Dot blot has similar technical features and specificity as western blot, but is the more sensitive method [6,7]. Currently dot blot is used in routine sample analysis [8-11], and at the same time

it has been proven to work well with proteins over a wide range of molecular weights [12]. Most of the dot blot methods which have been developed use only one developing signal [13,14]. The main disadvantage of the existing dot blot techniques, which limits their application, is the absence of signal normalization to a housekeeping protein. Stripping off previously used antibodies is one possibility to apply further antibodies for the detection of a housekeeping protein. However the stripping procedure often leads to a partial protein detachment from the membrane and thus to a signal lowering of the protein of interest.

In this study we established a one dot two development signals (ODTDS) dot blot method employing two different signal development systems. The first signal from the protein of interest was detected by horseradish peroxidase (HRP). The second signal, detecting the housekeeping protein, was obtained by using alkaline phosphatase (AP). Collagen I, nephrin, and pSMAD2/3 were used as proteins of interest whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein. We tested the efficiency of dot blot using urea/thiourea lysis buffer on kidney samples derived from sham-operated and 5/6 nephrectomized animals, which were taken from another study conducted by our group [15].

MATERIALS AND METHODS

Sample Preparation

Deep frozen pieces of 1/8 of the left kidney containing both medulla and cortex were mechanically turned into powder using a laboratory metal mortar and pestle precooled with liquid nitrogen. Urea/thiourea buffer [2 M thiourea, 7 M urea, 2% SDS, 1% DTT, and protease inhibitor (Complete Mini, Cat. No.: 11 697 498 001, Roche)] was used for protein extraction. Samples were sonicated on ice using the Bandelin Sonopuls GM 2070 sonicator (Bandelin electronic, Berlin, Germany) for 1.5 minutes (1 second on/1 second off cycles) at medium intensity, kept for 10 minutes at room temperature, centrifuged for 10 minutes at full speed. Afterwards, the supernatant was collected. Protein concentration was determined by spectrophotometry with Roti-Nanoquant (Cat. No. K800.1, Carl Roth). Standard samples were prepared using standard protein dilutions provided by the manufacturer.

Pure Protein Positive Controls

Pure protein positive control solutions were prepared by diluting pure collagen I from rat tail tendon (sc-136157, Santa Cruz Biotechnology, Inc., CA, USA) and pure GAPDH (ab77109, Abcam) as shown in Table 1 in urea/thiourea lysis buffer.

Animal models

Male Wistar rats (Charles River Laboratories International, Inc., Wilmington, MA, USA) were allocated into two groups: 5/6 nephrectomized (5/6 Nx) or sham operated. Surgery was performed as previously described [16].

Dot blot analysis

1 μ L urea/thiourea lysed samples at a concentration of 5 μ g/ μ L were spotted onto a nitrocellulose membrane (AmershamTM HybondTM ECL, GE Healthcare). The same procedure was applied for membranes, which were spotted with 0.125 μ g, 0.25 μ g, 0.5 μ g, and 1 μ g *pure protein positive control*. After complete drying for 5 minutes at room temperature, the membrane was washed with TBS-T three times for ten minutes.

The membranes were blocked in 5% non-fat milk/TBS-T (20 mM Tris, 140 mM NaCl, 0.1% Tween-20 at pH 7.6) for 1 hour at room temperature. First, the primary antibody incubation was performed overnight at +4°C. The anti-nephrin antibody (sc-28192, Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:2000, the anti-collagen I antibody (a gift from Prof. Schuppan [17]) at a dilution of 1:10000, the anti-pSMAD2/3 antibody (sc-11769-R, Santa Cruz Biotechnology, Inc.) at a dilution of 1:5000, and the anti-housekeeping protein GAPDH antibody (MAB 374, Millipore) at a dilution of 1:10000. Membranes were washed with TBS-T three times for ten minutes and incubated with HRP-conjugated secondary antibodies (sc-2054, Santa Cruz Biotechnology, Inc.). The signal was developed using enhanced chemiluminescence (ECL). Second, the same membranes were incubated with anti-GAPDH primary antibodies overnight at +4°C, washed three times in TBS-T and incubated with AP-conjugated secondary antibodies (sc-2008, Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. The signal was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT) substrate. The developed membranes were digitalized using a 600 dpi scanner resolution. Quantitative analysis was performed using AlphaEaseFCTM software version 3.1.2. (Alpha Innotech Corporation). The signal protein expression was determined using integrated density value (IDV). The background expression was determined individually for every given sample and was subtracted from the main signal. The final results were calculated as a ratio between the protein of interest expression and GAPDH. A standard sample was constituted by mixing aliquots of all samples.

Western Blot Analysis

15 μ g protein extract was loaded into each separation well of the gel. 10% polyacrylamide gels were used for SDS-PAGE. After SDS-PAGE gels were blotted to nitrocellulose membrane (AmershamTM HybondTM ECL, GE Healthcare) using a Biorad Trans-Blot semidry blotter and transfer buffer (184 mM glycine, 24 mM Tris,

Table 1. Pure Protein Positive Control Composition.

	Pure Protein Positive Control	Collagen I concentration (µg/µL)	GAPDH concentration (µg/µL)
Collagen I added GAPDH	Col I with 20% GAPDH	1	0.2
	Col I with 5% GAPDH	1	0.05
	Col I with 1% GAPDH	1	0.01
GAPDH added collagen I	GAPDH with 20% Col I	0.2	1
	GAPDH with 5% Col I	0.05	1
	GAPDH with 1% Col I	0.01	1

Table 2. Linear regression analysis of dot blot standard curves.

Protein of Interest	Corresponding GAPDH
pSMAD2/3: $y = 186110x - 205622$ $R^2 = 0.9850$	$y = 161619x - 199205$ $R^2 = 0.9785$
Collagen I: $y = 244643x + 398354$ $R^2 = 0.9652$	$y = 35663x - 34914$ $R^2 = 0.9797$
Nephrin: $y = 1052,2x - 435,07$ $R^2 = 0.9879$	$y = 15708x - 1753,5$ $R^2 = 0.9877$

x - protein amount (µg), y - IDV (Integrated DensityValue).

Table 3. Inter-assay results variations within ODTDS dot blot and western blot and intra-assay variations between both methods.

Protein	Inter-assay		Intra-assay
	WB	ODTDS Dot Blot	WB and ODTDS Dot Blot
Collagen I	1.04 ± 0.98	1.55 ± 2.00	1.72 ± 1.71
pSMAD2/3	2.16 ± 2.18	2.78 ± 2.84	5.49 ± 5.31
Nephrin	2.94 ± 2.87	3.24 ± 2.53	5.71 ± 7.29

Coefficient of variation in percent. The values are given as mean ± SD.

20% methanol) at 25 V for 1 hour. All subsequent steps were exactly the same as in the dot blot.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 software. Student's *t*-test was performed if the variables were parametric and normally distributed, as determined by the Kolmogorov-Smirnov test; otherwise, the Mann-Whitney U-test was used. A confidence interval of 0.05 was used. Coefficient of variation (CV)

was determined as the ratio of the standard deviation to the mean value of protein signal. Linear regression analysis was performed to prove that IDV signal origins were from the protein of interest and not from the background signal.

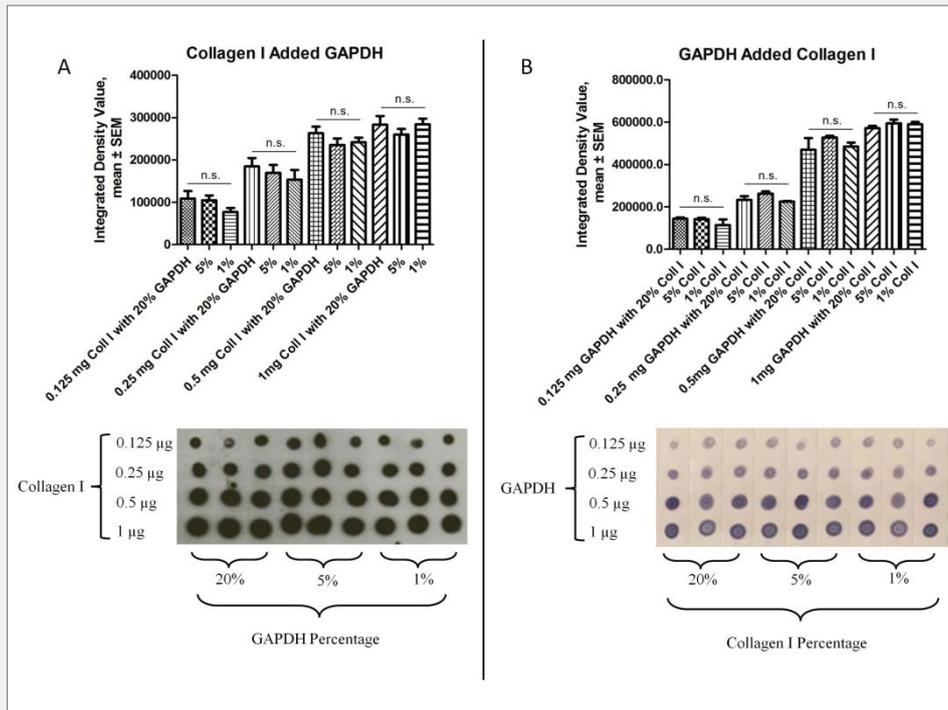


Figure 1. There was no significant difference (n.s.) between the signal from 0.125 µg collagen I added with 1%, 5%, and 20% GAPDH. The same results were obtained in the case of 0.25 µg, 0.5 µg, and 1 µg collagen I with added GAPDH samples (A). Addition of collagen I also had no impact on the GAPDH signal (B).

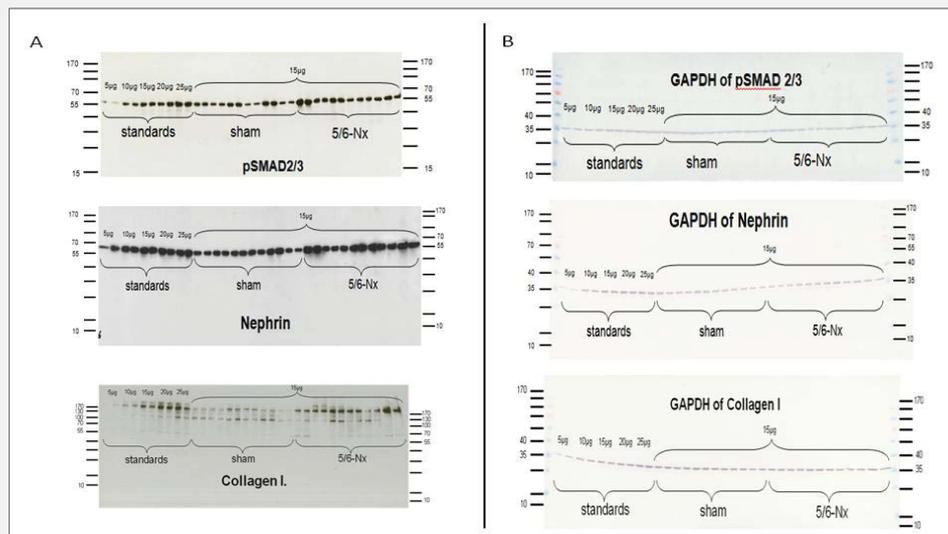


Figure 2. Western blot for pSMAD2/3, nephrin, collagen I (A) and corresponding GAPDH (B).

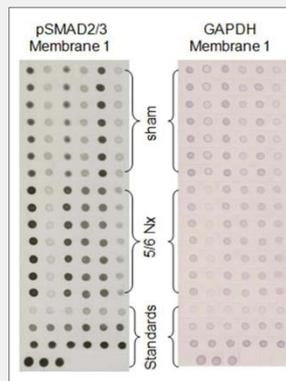


Figure 3. A typical result from ODTDS dot blot method. Six sham and 5/6 Nx protein samples were spotted in heptaplicates in the amount of 5 μ g. Standard protein was spotted in triplicates in the amounts of 2.1, 2.8, 3.8, 5, 7.5, 10, and 20 μ g per dot.

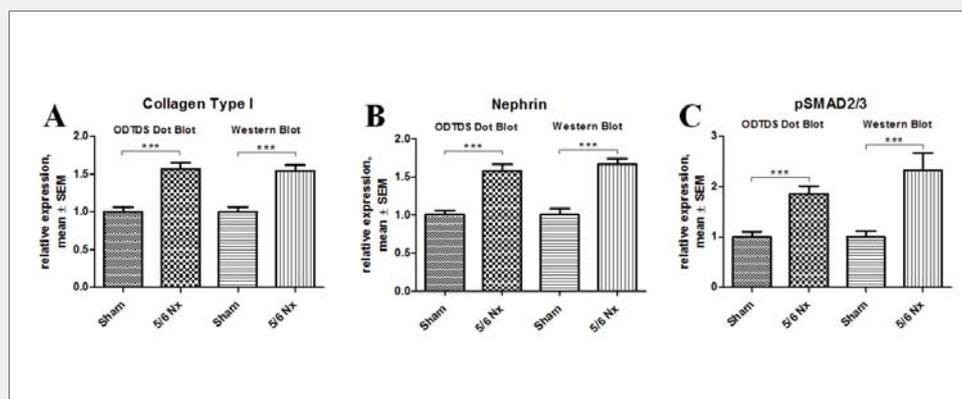


Figure 4. Relative protein expression of collagen type I (A), nephrin (B) and pSMAD2/3 in whole kidney assessed by ODTDS dot blot and western blot methods.

Values are given as mean \pm SEM; *** - $p < 0.001$.

RESULTS

Pure protein positive control samples were prepared to investigate interplay correlation of the first and second signal development system in the same blot. The presence of collagen I in the sample (1%, 5%, and 20%) had no impact on the signal intensity obtained from the added amount of GAPDH (0.125 μ g; 0.25 μ g; 0.5 μ g; 1 μ g, Figure 1a). The same results were observed for samples of collagen I with added amounts of GAPDH (Figure 1b).

This proves that complexes of *collagen I-anti collagen I antibody-secondary antibody* did not impact the devel-

opment of the alkaline phosphatase signal of *GAPDH-anti GAPDH antibody-secondary antibody* complexes. AP signal development for detection of GAPDH-anti GAPDH-secondary antibody was not impaired by complex formations of collagen I-anti collagen I antibody-secondary antibody.

As shown in Figure 1, there was no significant difference (n. s.) between the signal from 0.125 μ g collagen I added with 1%, 5%, and 20% GAPDH. The same results were obtained for 0.25 μ g, 0.5 μ g, and 1 μ g collagen I with added amounts of GAPDH (A). Addition of collagen I also had no impact on the GAPDH signal (B).

Protein extract samples of normal and 5/6 nephrectomized rat kidneys were used to test the ODTDS dot blot method in comparison to western blot regarding the detection of kidney biomarkers. Western blot was used as comparison, as it is the best established method to investigate up/down-regulation of selected biomarkers. Western blot was also performed to ensure that the signal given by ODTDS dot blot method came only from the protein of interest. Phosphorylated SMAD2/3, nephrin, and GAPDH gave a single band at about 57 kDa, 70 kDa [18], and 37 kDa, respectively. Collagen I gave three different bands, one at about 70 kDa representing mature collagen [19,20], and two bands at 130 kDa and 190 kDa representing collagen I precursors [21] (Figure 2).

The calibration curve for the ODTDS dot blot method was built based on 2.1, 2.8, 3.8, 5, 7.5, 10, and 20 μ g standard protein triplicates and linearity coefficients (R^2) from 0.965 to 0.988 were observed (Table 2). In order to get the best results for the ODTDS dot blot method, 5 μ g protein per dot was used for all proteins of interest and the typical result is presented in Figure 3. The coefficient of variation (CV) is widely used as an indicator of a newly established method performance [22]. In the current study, CV was used to investigate the inter-assay results variations within the ODTDS dot blot and western blot as well as intra-assay variations between both methods (Table 3). Inter-assay variation between the ODTDS method results gave CVs between 1.55% and 3.24% for all detected proteins. Evaluation of the ODTDS method in regard to western blot gave CVs between 1.72% and 5.71%.

The differences in protein expression levels between the study groups were found to be the same as measured by both ODTDS dot blot and western blot methods. Furthermore, the statistical differences between the groups as elucidated by p value, were at the same level of $p < 0.001$ (Figure 4).

DISCUSSION

Western blot is the most common method for evaluating and quantifying protein expression; however, it is very labor intensive and time consuming when dealing with large sample sizes.

Since dot blot has almost the same reaction scheme as western blot, except for the protein separation step (SDS-PAGE), this method is frequently chosen as a replacement for western blot. Some routine dot blots have been developed to replace western blot and to standardize dot blot for large sample sizes [6,8]. However, to the best of our knowledge, there is no report about signal normalization of dot blot results. Since pipetting mistakes are quite common during a dot blot procedure, it is very important to develop signal normalization of the dot blot results. To address this we developed the ODTDS (One Dot Two Development Signals) dot blot method.

Steric hindrance is a potential problem in developing ODTDS because the first complex antigen 1 - primary antibody 1 - secondary antibody 1 may block or reduce the formation of the second complex antigen 2 - primary antibody 2 - secondary antibody 2. In this study collagen I was used to test for the aforementioned potential problem. Collagen I belongs to a class of heavy molecular weight proteins and accordingly has a higher blocking potential. GAPDH was chosen because it is a housekeeping gene that is often used to normalize the results of the western blot signal. The comparison of GAPDH signals from samples containing 1%, 5%, and 20% collagen type I gave no significant differences (*t*-test, 95% confidence interval), indicating no steric hindrance of collagen I on the development of the GAPDH signal. The same result was observed for collagen I added with 1%, 5%, and 20% GAPDH. Guillemin et al. (2009) observed the same result when using a conventional dot blot to detect other proteins which have variations in their molecular weight, cellular abundance, and dimensional structure [6]. Our data suggest that the ODTDS dot blot method can be used to analyze the expression level of proteins of interest and also to normalize the signal using GAPDH.

Linear response is a very critical approach due to the importance of recalibrating the developing method [23, 25]. The linearity of the signal derived from protein standards used in the ODTDS dot blot method was confirmed by high linearity coefficients (0.965 to 0.988, Table 2). This fact indicates that the signal was derived from the same protein species even though the total protein standard amount was increased, or in other words, we excluded the signal coming from background.

Statistical testing is frequently used to evaluate a newly established method. For this purpose the coefficient of variation (CV) was used to examine the experimental results for absence of technical biases. In this study, inter-assay CV values of the ODTDS dot blot method were 1.55 - 3.24% and the intra-assay CV values with western blot were 1.72 - 5.71% (Table 3). Moreover, inter-assay CV values of the ODTDS dot blot were almost in the same range compared to the ones for western blot (1.04 - 2.94%). CV values below 10% are known to be a characteristic of a good method [26].

Conventional dot blot method belongs to a yes/no assay type [8,27]. Častorálová et al. used dot blot for quantification of protein expression, although this was done without signal normalization to a housekeeping protein [12].

Guillemin et al. (2009) showed that conventional dot blot is as good as western blot for use as a quantitative method for measuring protein expression [6]. However, there is no evidence about using the same blot to develop two different signals in a dot blot method. Herein, we introduce the ODTDS dot blot method as a quantitative assay for measurement of single protein of interest expression. In the method we established, the pipetting inaccuracies are normalized by measuring a housekeeping protein with another detection system on the same

blot. We clearly showed that the ODTDS dot blot method is as good as western blot to examine expression of certain proteins. As a new variant of the dot blot method, ODTDS has no/minimal technical biases and can be used in large numbers of samples since it is simple and fast to apply.

Declaration of Interest:

There is no conflict of interest for any of the authors.

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ORIGINAL ARTICLE

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**Dealing with Large Sample Sizes:
Comparison of a New One Spot Dot Blot Method to Western Blot**

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SUMMARY

Background: Western blot is the gold standard method to determine individual protein expression levels. However, western blot is technically difficult to perform in large sample sizes because it is a time consuming and labor intensive process. Dot blot is often used instead when dealing with large sample sizes, but the main disadvantage of the existing dot blot techniques, is the absence of signal normalization to a housekeeping protein.

Methods: In this study we established a one dot two development signals (ODTDS) dot blot method employing two different signal development systems. The first signal from the protein of interest was detected by horseradish peroxidase (HRP). The second signal, detecting the housekeeping protein, was obtained by using alkaline phosphatase (AP).

Results: Inter-assay results variations within ODTDS dot blot and western blot and intra-assay variations between both methods were low (1.04 - 5.71%) as assessed by coefficient of variation.

Conclusions: ODTDS dot blot technique can be used instead of western blot when dealing with large sample sizes without a reduction in results accuracy.

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KEY WORDS

one dot two development signals (ODTDS) dot blot, western blot, protein quantification, large sample size studies, comparison

INTRODUCTION

Western blot is the gold standard method to determine individual protein expression levels. However, western blot is technically difficult to perform in large sample sizes because it is a time consuming and labor intensive process. Dot blot is often used instead when dealing with large sample sizes [1-5]. Dot blot has similar technical features and specificity as western blot, but is the more sensitive method [6,7]. Currently dot blot is used in routine sample analysis [8-11], and at the same time

it has been proven to work well with proteins over a wide range of molecular weights [12]. Most of the dot blot methods which have been developed use only one developing signal [13,14]. The main disadvantage of the existing dot blot techniques, which limits their application, is the absence of signal normalization to a housekeeping protein. Stripping off previously used antibodies is one possibility to apply further antibodies for the detection of a housekeeping protein. However the stripping procedure often leads to a partial protein detachment from the membrane and thus to a signal lowering of the protein of interest.

In this study we established a one dot two development signals (ODTDS) dot blot method employing two different signal development systems. The first signal from the protein of interest was detected by horseradish peroxidase (HRP). The second signal, detecting the housekeeping protein, was obtained by using alkaline phosphatase (AP). Collagen I, nephrin, and pSMAD2/3 were used as proteins of interest whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein. We tested the efficiency of dot blot using urea/thiourea lysis buffer on kidney samples derived from sham-operated and 5/6 nephrectomized animals, which were taken from another study conducted by our group [15].

MATERIALS AND METHODS

Sample Preparation

Deep frozen pieces of 1/8 of the left kidney containing both medulla and cortex were mechanically turned into powder using a laboratory mortar and pestle pre-cooled with liquid nitrogen. Urea/thiourea buffer [2 M thiourea, 7 M urea, 2% SDS, 1% DTT, and protease inhibitor (Complete Mini, Cat. No.: 11 697 498 001, Roche)] was used for protein extraction. Samples were sonicated on ice using the Bandelin Sonopuls GM 2070 sonicator (Bandelin electronic, Berlin, Germany) for 1.5 minutes (1 second on/1 second off cycles) at medium intensity, kept for 10 minutes at room temperature, centrifuged for 10 minutes at full speed. Afterwards, the supernatant was collected. Protein concentration was determined by spectrophotometry with Roti-Nanoquant (Cat. No. K800.1, Carl Roth). Standard samples were prepared using standard protein dilutions provided by the manufacturer.

Pure Protein Positive Controls

Pure protein positive control solutions were prepared by diluting pure collagen I from rat tail tendon (sc-136157, Santa Cruz Biotechnology, Inc., CA, USA) and pure GAPDH (ab77109, Abcam) as shown in Table 1 in urea/thiourea lysis buffer.

Animal models

Male Wistar rats (Charles River Laboratories International, Inc., Wilmington, MA, USA) were allocated into two groups: 5/6 nephrectomized (5/6 Nx) or sham operated. Surgery was performed as previously described [16].

Dot blot analysis

1 μ L urea/thiourea lysed samples at a concentration of 5 μ g/ μ L were spotted onto a nitrocellulose membrane (Amersham™ Hybond™ ECL, GE Healthcare). The same procedure was applied for membranes, which were spotted with 0.125 μ g, 0.25 μ g, 0.5 μ g, and 1 μ g pure protein positive control. After complete drying for 5 minutes at room temperature, the membrane was washed with TBS-T three times for ten minutes.

The membranes were blocked in 5% non-fat milk/TBS-T (20 mM Tris, 140 mM NaCl, 0.1% Tween-20 at pH 7.6) for 1 hour at room temperature. First, the primary antibody incubation was performed overnight at +4°C. The anti-nephrin antibody (sc-28192, Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:2000, the anti-collagen I antibody (a gift from Prof. Schuppan [17]) at a dilution of 1:10000, the anti-pSMAD2/3 antibody (sc-11769-R, Santa Cruz Biotechnology, Inc.) at a dilution of 1:5000, and the anti-housekeeping protein GAPDH antibody (MAB 374, Millipore) at a dilution of 1:10000. Membranes were washed with TBS-T three times for ten minutes and incubated with HRP-conjugated secondary antibodies (sc-2054, Santa Cruz Biotechnology, Inc.). The signal was developed using enhanced chemiluminescence (ECL). Second, the same membranes were incubated with anti-GAPDH primary antibodies overnight at +4°C, washed three times in TBS-T and incubated with AP-conjugated secondary antibodies (sc-2008, Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. The signal was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT) substrate. The developed membranes were digitalized using a 600 dpi scanner resolution. Quantitative analysis was performed using AlphaEaseFC™ software version 3.1.2. (Alpha Innotech Corporation). The signal protein expression was determined using integrated density value (IDV). The background expression was determined individually for every given sample and was subtracted from the main signal. The final results were calculated as a ratio between the protein of interest expression and GAPDH. A standard sample was constituted by mixing aliquots of all samples.

Western Blot Analysis

15 μ g protein extract was loaded into each separation well of the gel. 10% polyacrylamide gels were used for SDS-PAGE. After SDS-PAGE gels were blotted to nitrocellulose membrane (Amersham™ Hybond™ ECL, GE Healthcare) using a Biorad Trans-Blot semidry blotter and transfer buffer (184 mM glycine, 24 mM Tris,

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Table 1. Pure Protein Positive Control Composition.

	Pure Protein Positive Control	Collagen I concentration (µg/µL)	GAPDH concentration (µg/µL)
Collagen I added GAPDH	Col I with 20% GAPDH	1	0.2
	Col I with 5% GAPDH	1	0.05
	Col I with 1% GAPDH	1	0.01
GAPDH added collagen I	GAPDH with 20% Col I	0.2	1
	GAPDH with 5% Col I	0.05	1
	GAPDH with 1% Col I	0.01	1

Table 2. Linear regression analysis of dot blot standard curves.

Protein of Interest	Corresponding GAPDH
pSMAD2/3: $y = 186110x - 205622$ $R^2 = 0.9850$	$y = 161619x - 199205$ $R^2 = 0.9785$
Collagen I: $y = 244643x + 398354$ $R^2 = 0.9652$	$y = 35663x - 34914$ $R^2 = 0.9797$
Nephrin: $y = 1052,2x - 435,07$ $R^2 = 0.9879$	$y = 15708x - 1753,5$ $R^2 = 0.9877$

x - protein amount (µg), y - IDV (Integrated Density Value).

Table 3. Inter-assay results variations within ODTDS dot blot and western blot and intra-assay variations between both methods.

Protein	Inter-assay		Intra-assay
	WB	ODTDS Dot Blot	WB and ODTDS Dot Blot
Collagen I	1.04 ± 0.98	1.55 ± 2.00	1.72 ± 1.71
pSMAD2/3	2.16 ± 2.18	2.78 ± 2.84	5.49 ± 5.31
Nephrin	2.94 ± 2.87	3.24 ± 2.53	5.71 ± 7.29

Coefficient of variation in percent. The values are given as mean ± SD.

20% methanol) at 25 V for 1 hour. All subsequent steps were exactly the same as in the dot blot.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 software. Student's *t*-test was performed if the variables were parametric and normally distributed, as determined by the Kolmogorov-Smirnov test; otherwise, the Mann-Whitney U-test was used. A confidence interval of 0.05 was used. Coefficient of variation (CV)

was determined as the ratio of the standard deviation to the mean value of protein signal. Linear regression analysis was performed to prove that IDV signal origins were from the protein of interest and not from the background signal.

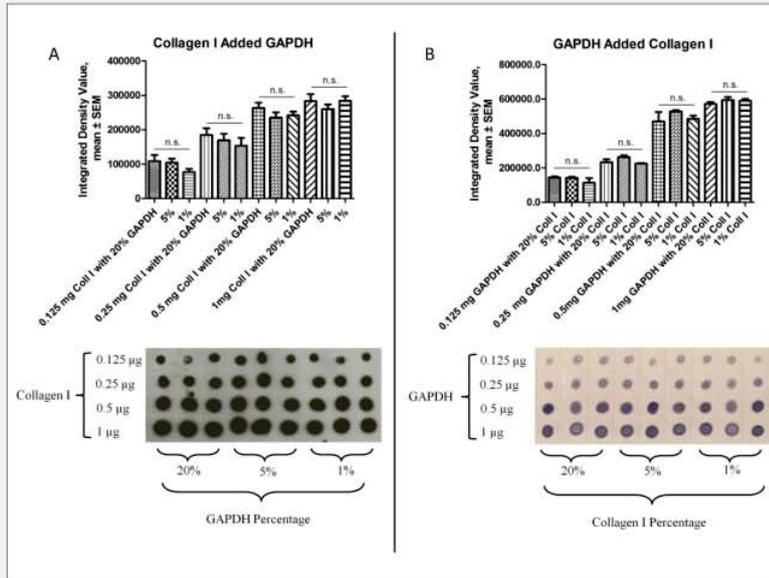


Figure 1. There was no significant difference (n.s.) between the signal from 0.125 µg collagen I added with 1%, 5%, and 20% GAPDH. The same results were obtained in the case of 0.25 µg, 0.5 µg, and 1 µg collagen I with added GAPDH samples (A). Addition of collagen I also had no impact on the GAPDH signal (B).

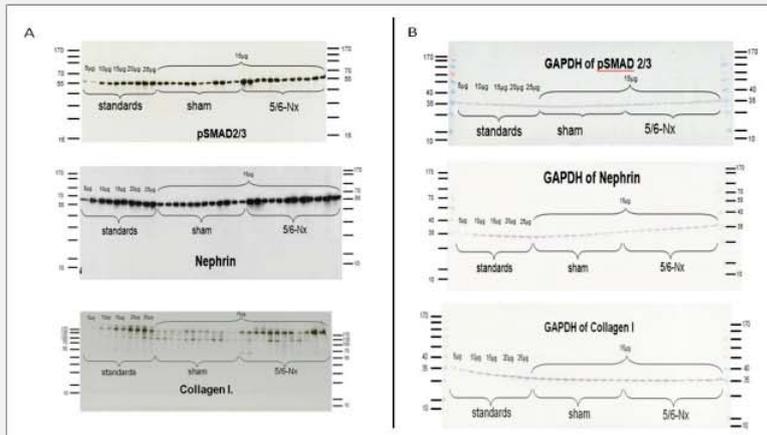


Figure 2. Western blot for pSMAD2/3, nephrin, collagen I (A) and corresponding GAPDH (B).

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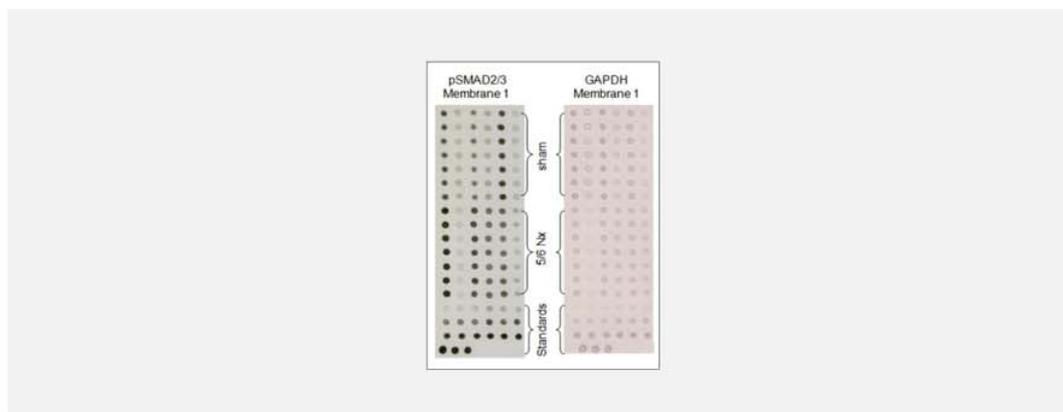


Figure 3. A typical result from ODTDS dot blot method. Six sham and 5/6 Nx protein samples were spotted in heptaplicates in the amount of 5 μ g. Standard protein was spotted in triplicates in the amounts of 2.1, 2.8, 3.8, 5, 7.5, 10, and 20 μ g per dot.

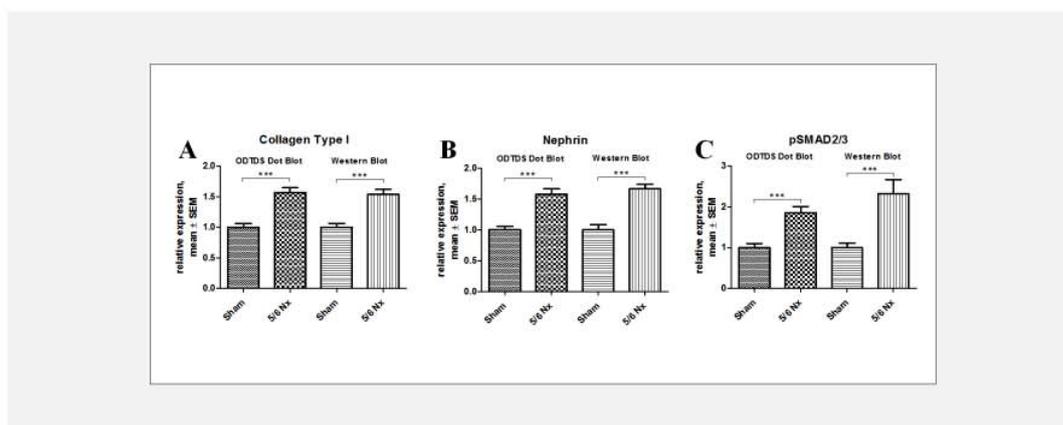


Figure 4. Relative protein expression of collagen type I (A), nephrin (B) and pSMAD2/3 in whole kidney assessed by ODTDS dot blot and western blot methods.

Values are given as mean \pm SEM; *** - $p < 0.001$.

RESULTS

Pure protein positive control samples were prepared to investigate interplay correlation of the first and second signal development system in the same blot. The presence of collagen I in the sample (1%, 5%, and 20%) had no impact on the signal intensity obtained from the added amount of GAPDH (0.125 μ g; 0.25 μ g; 0.5 μ g; 1 μ g, Figure 1a). The same results were observed for samples of collagen I with added amounts of GAPDH (Figure 1b).

This proves that complexes of collagen I-anti collagen I antibody-secondary antibody did not impact the devel-

opment of the alkaline phosphatase signal of GAPDH-anti GAPDH antibody-secondary antibody complexes. AP signal development for detection of GAPDH-anti GAPDH-secondary antibody was not impaired by complex formations of collagen I-anti collagen I antibody-secondary antibody.

As shown in Figure 1, there was no significant difference (n. s.) between the signal from 0.125 μ g collagen I added with 1%, 5%, and 20% GAPDH. The same results were obtained for 0.25 μ g, 0.5 μ g, and 1 μ g collagen I with added amounts of GAPDH (A). Addition of collagen I also had no impact on the GAPDH signal (B).

Protein extract samples of normal and 5/6 nephrectomized rat kidneys were used to test the ODTDS dot blot method in comparison to western blot regarding the detection of kidney biomarkers. Western blot was used as comparison, as it is the best established method to investigate up/down-regulation of selected biomarkers. Western blot was also performed to ensure that the signal given by ODTDS dot blot method came only from the protein of interest. Phosphorylated SMAD2/3, nephrin, and GAPDH gave a single band at about 57 kDa, 70 kDa [18], and 37 kDa, respectively. Collagen I gave three different bands, one at about 70 kDa representing mature collagen [19,20], and two bands at 130 kDa and 190 kDa representing collagen I precursors [21] (Figure 2).

The calibration curve for the ODTDS dot blot method was built based on 2.1, 2.8, 3.8, 5, 7.5, 10, and 20 μg standard protein triplicates and linearity coefficients (R^2) from 0.965 to 0.988 were observed (Table 2). In order to get the best results for the ODTDS dot blot method, 5 μg protein per dot was used for all proteins of interest and the typical result is presented in Figure 3. The coefficient of variation (CV) is widely used as an indicator of a newly established method performance [22]. In the current study, CV was used to investigate the inter-assay results variations within the ODTDS dot blot and western blot as well as intra-assay variations between both methods (Table 3). Inter-assay variation between the ODTDS method results gave CVs between 1.55% and 3.24% for all detected proteins. Evaluation of the ODTDS method in regard to western blot gave CVs between 1.72% and 5.71%.

The differences in protein expression levels between the study groups were found to be the same as measured by both ODTDS dot blot and western blot methods. Furthermore, the statistical differences between the groups as elucidated by p value, were at the same level of $p < 0.001$ (Figure 4).

DISCUSSION

Western blot is the most common method for evaluating and quantifying protein expression; however, it is very labor intensive and time consuming when dealing with large sample sizes.

Since dot blot has almost the same reaction scheme as western blot, except for the protein separation step (SDS-PAGE), this method is frequently chosen as a replacement for western blot. Some routine dot blots have been developed to replace western blot and to standardize dot blot for large sample sizes [6,8]. However, to the best of our knowledge, there is no report about signal normalization of dot blot results. Since pipetting mistakes are quite common during a dot blot procedure, it is very important to develop signal normalization of the dot blot results. To address this we developed the ODTDS (One Dot Two Development Signals) dot blot method.

Steric hindrance is a potential problem in developing ODTDS because the first complex antigen 1 - primary antibody 1 - secondary antibody 1 may block or reduce the formation of the second complex antigen 2 - primary antibody 2 - secondary antibody 2. In this study collagen I was used to test for the aforementioned potential problem. Collagen I belongs to a class of heavy molecular weight proteins and accordingly has a higher blocking potential. GAPDH was chosen because it is a housekeeping gene that is often used to normalize the results of the western blot signal. The comparison of GAPDH signals from samples containing 1%, 5%, and 20% collagen type I gave no significant differences (*t*-test, 95% confidence interval), indicating no steric hindrance of collagen I on the development of the GAPDH signal. The same result was observed for collagen I added with 1%, 5%, and 20% GAPDH. Guillemín et al. (2009) observed the same result when using a conventional dot blot to detect other proteins which have variations in their molecular weight, cellular abundance, and dimensional structure [6]. Our data suggest that the ODTDS dot blot method can be used to analyze the expression level of proteins of interest and also to normalize the signal using GAPDH.

Linear response is a very critical approach due to the importance of recalibrating the developing method [23, 25]. The linearity of the signal derived from protein standards used in the ODTDS dot blot method was confirmed by high linearity coefficients (0.965 to 0.988, Table 2). This fact indicates that the signal was derived from the same protein species even though the total protein standard amount was increased, or in other words, we excluded the signal coming from background.

Statistical testing is frequently used to evaluate a newly established method. For this purpose the coefficient of variation (CV) was used to examine the experimental results for absence of technical biases. In this study, inter-assay CV values of the ODTDS dot blot method were 1.55 - 3.24% and the intra-assay CV values with western blot were 1.72 - 5.71% (Table 3). Moreover, inter-assay CV values of the ODTDS dot blot were almost in the same range compared to the ones for western blot (1.04 - 2.94%). CV values below 10% are known to be a characteristic of a good method [26].

Conventional dot blot method belongs to a yes/no assay type [8,27]. Častorálová et al. used dot blot for quantification of protein expression, although this was done without signal normalization to a housekeeping protein [12].

Guillemín et al. (2009) showed that conventional dot blot is as good as western blot for use as a quantitative method for measuring protein expression [6]. However, there is no evidence about using the same blot to develop two different signals in a dot blot method. Herein, we introduce the ODTDS dot blot method as a quantitative assay for measurement of single protein of interest expression. In the method we established, the pipetting inaccuracies are normalized by measuring a housekeeping protein with another detection system on the same

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blot. We clearly showed that the ODTDS dot blot method is as good as western blot to examine expression of certain proteins. As a new variant of the dot blot method, ODTDS has no/minimal technical biases and can be used in large numbers of samples since it is simple and fast to apply.

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Declaration of Interest:

There is no conflict of interest for any of the authors.

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