



## Original Research Article

## A pilot study to compare urinary protein of fractional urine collected over 24h period versus traditional 24h urine collection method

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## ABSTRACT

**Introduction:** 24 hours traditional urine collection method is cumbersome and tedious process with less patient compliance. Our study proposes a novel fractional urine collection method for protein estimation that would entail collection of small volumes of urine each time patient voids Over 24h Period. Our aim was to compare urine protein in proposed method versus traditional 24hour sampling method.

**Materials and Methods:** Cross-sectional, prospective study was a collaborative work between Departments of Nephrology and Biochemistry in our tertiary care hospital. Volunteers (48) and Chronic Kidney disease subjects (52) were recruited. Out of which, 76 subjects were selected. Novel method was compared with conventional method in each of these groups, before and after centrifugation and with and without preservative. Intraclass correlation coefficient (ICC) and Bland Altman analysis was used to evaluate for agreement between the two methods.

**Results:** All the values were combined without categorization into subgroups. Fractional urine collection method (before centrifugation) without preservative (F1) and with preservative (F2) was compared to conventional method, ICC was 0.93 (C.I, 0.9 to 0.95) and 0.96 (C.I, 0.94 to 0.97) respectively. After centrifugation, fractional urine collection method for F1 and F2 versus conventional method, ICC was 0.95 (C.I, 0.92 to 0.97) and 0.91 (C.I, 0.86 to 0.94) respectively.

**Conclusions:** The novel collection method is comparable and reliable as traditional 24-hour method. The use of thymol as preservative leads to negative interference and centrifugation is mandatory before urine analysis. With proper sampling, urine protein by novel method has a good agreement with the conventional technique.

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### 1. Introduction

The urine protein excretion is a sensitive marker that reflects varying stages of kidney disease from an early to advanced stage. Estimation of 24-hour (24h) urine protein has been considered as gold standard for quantitation of proteinuria. However, 24h urine collection has several disadvantages such as failure to collect the entire urine volume during collection period besides the process itself being inconvenient, cumbersome, storage difficulties and need of preservative. All these pre-analytical issues impact

the evaluation of proteinuria.<sup>1</sup> Several studies<sup>2–4</sup> have been reported supporting shorter timed samples to reduce or minimize the pre-analytical errors.

Alternatively, concurrent measure of Creatinine in the sample was assumed to provide completeness of collection and Protein to Creatinine ratio (PCR) has been deemed to be an accurate measure to evaluate the extent of proteinuria. However, there is wide confidence Interval (CI) with poorer correlation in nephrotic range proteinuria and patients with low Glomerular Filtration Rate (GFR).<sup>5–9</sup>

Nayak et al<sup>10</sup> in their attempt to study the accuracy of spot urine protein creatinine ratio in measuring proteinuria

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in Chronic Kidney disease patients of stage 3 and 4 have shown that by using correction factor for creatinine to calculate 24hUrine Creatinine improved the accuracy of spot urine protein creatinine ratio. There were limitations in terms of data being more representative of males and focused on South Indian (South Asian) population.

In view of these challenges, this study was aimed to improve the conventional or traditional collection method by new method that entails collecting a fraction of urine sample with every void over 24h Period and compared parameters with the conventional method. We hypothesized that there is no difference in parameters measured in fractional urine collection over a period of 24h versus the conventional method.

### 1.1. Subjects and methods

#### 1.2. Objectives of the study

The primary objective of the study is to compare and evaluate urine protein values in samples collected by the fractional method and conventional method.

#### 1.3. The secondary objective was

1. To compare and evaluate the effect of centrifugation on urine protein values in urine samples collected by fractional and conventional method.
2. To compare and evaluate the effect of thymol (preservative for protein) on urine protein values in urine samples collected by fractional and conventional method.

## 2. Materials and Methods

### 2.1. Study design and participants

This cross-sectional, prospective study was carried out as a collaborative work between Departments of Nephrology and Biochemistry in our tertiary care hospital. After obtaining Institutional Ethical Clearance and formal consent, we recruited 100 adult subjects for the study. Participants included volunteers and subjects who attended the Out-Patient clinic during a study period of one year from October 2016 to September 2017. Participants belonging to age group between 18 and 60 years, either gender, with no history of urinary incontinence, Individuals not on urinary catheter or undergoing dialysis, non pregnant and lactating women and not having menstruation at the time of collection were included in the study. Improper collection or not complying with collection protocol, accidental spillage during collection period or at any time before it's measurement for volume /parameter, or suffering from gastroenteritis were excluded.

Demographic data and detailed medical history were recorded in a pre-designed data sheet at the time of

recruitment of participants for the study.

### 2.2. Protocol for 24h urine collection

Subjects were provided with detailed instructions along with schematic diagram for 24h urine collection. As per the protocol, urine collection starts in the morning, after the first void till the same time next morning (for example - at 7am after discarding first morning void, till 7am on the next day). Participants were provided with 3 (one large and two small) closed containers, a jar, disposable syringes. Large container was meant for 24h urine collection by traditional method without preservative, labeled as 24 h while the two small containers were labeled as F1 (without preservative) and F2 (with preservative thymol) for urine collection by proposed method.

Subjects were instructed to pass urine into the clean jar every time they wanted to void. 1 mL of urine was transferred to F1 and F2 each, before the entire content in the jar was transferred to 24h container. Jar was cleaned, dried and kept aside for the next void. This process was repeated till the end of collection period of 24h. to protocol for urine sampling and analysis

Sample volume were measured in the three containers (24h, F1 and F2). Two aliquots of 2.0 mL each were taken from all the three containers after mixing the samples thoroughly. One set of aliquots from every container was centrifuged and supernatant was aliquoted. It was labeled as 24 ha, F1a and F2 a. The other set of aliquots (without centrifugation) were labeled as 24 hb, F1b, F2b.

Remaining urine samples in F1 and F2 were mixed well and transferred to 24 h container. Two aliquots of 2.0 mL each were taken after thoroughly mixing the contents and labeled as C-24h a (after centrifugation) and C-24hb (before centrifugation).

All urine aliquots were analyzed for protein by using Pyrogallol Red photometric method in Siemens Dimension RxL by Siemens Healthcare Pvt. Ltd. Urine Creatinine was measured in 24 h sample using Modified Jaffe's method, standardized to IDMS method in Siemens Dimension Healthcare Pvt. Ltd. to assess urine creatinine excretion (UCE).

To evaluate for completeness of 24 h urine collection, Creatinine Index was used. It is ratio of observed UCE to Expected UCE and ratio of less than 0.6 was assumed as potentially incomplete 24 h urine collection.<sup>11</sup>

### 2.3. Statistical analysis

Descriptive statistics was used to describe the demographic data and variables such as urine volume, protein concentration in various containers. Samples were described as Mean  $\pm$  Standard Deviation (SD). Bland-Altman Analysis were done to evaluate the extent of agreement between the two methods in different groups. Extent of agreement

was shown as a mean difference along with their limits. Intraclass correlation was used to evaluate samples grouped based on centrifugation and preservative use.

### 3. Results

100 Subjects (n = 56 males, 44 females) were recruited to be evaluated for new method that entails fractional urine collection and compared to conventional urine collection method over 24h period. 48 volunteers were apparently healthy subjects with no co-morbidities while 52 subjects were cases of Chronic Kidney disease (CKD). All of them were evaluated for urine protein and creatinine. Out of which, 76 subjects were selected for the study while 24 subjects were excluded from the study since they did not follow the collection protocol. 45 samples in the CKD were predominantly diabetic nephropathy (n=20) followed by chronic glomerulonephritis (n=10) and others included lupus nephritis (n=5), hypertensive nephrosclerosis (n=5) and nephrotic syndrome (n=5). Table 1 details the demographic data collected at the time of study.

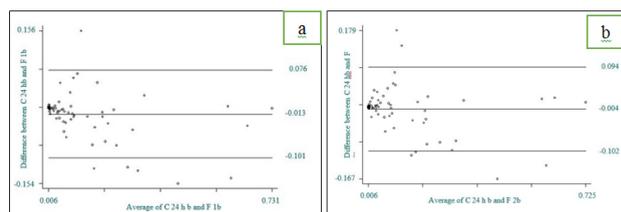
Further the subjects were grouped into three categories based on protein excretion per day<sup>12</sup>. Group 1 with protein excretion of less than 0.3 g/day; group 2 with protein excretion between 0.3 g/day to 1g/day while group 3 with protein excretion greater than 1 g/day. Fractional urine collection method was compared with conventional method in each of these groups.

Group 1 (Protein excretion less than 0.3 g/day) data seems skewed since majority of apparently healthy volunteers had urine protein values spanning around 0.006 g/dL (or 6 mg/dL) while only 7 subjects had values greater than 0.010 g/dL (or 10 mg/dL). Group 2 (protein excretion between 0.3 g/day and 1.0 g/day) data was normally distributed. Group 3 (protein excretion greater than 1.0 g/day) had protein values that spread over a large range and hence, a skewed distribution. The actual values obtained during protein estimation (g/dL) has been used for comparison, since the protein values in g/day may be misleading due to variance in urine volume collection, though categories have been made based on the daily excretion.

Protein values (g/dL) in conventional and fractional method (with and without preservative) is detailed in Tables 2 and 3 gives details on the mean difference and limits of agreements in fractional and conventional method. Effect of centrifugation and preservative on fractional urine collection method is provided in Table 4

Overall, when all the values are combined without categorization into groups, comparison of fractional urine collection method without (F1) and with (F2) preservative versus conventional method, Intra class coefficient (ICC) before centrifugation was 0.93 (C.I, 0.9 to 0.95) and 0.96 (C.I, 0.94 to 0.97) respectively, which is indicative of good correlation while, ICC after centrifugation was 0.95 (C.I,

0.92 to 0.97) and 0.91 (C.I, 0.86 to 0.94) respectively, which is indicative of good correlation.



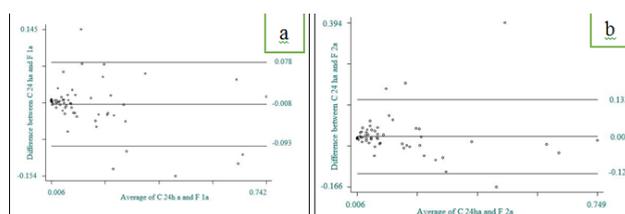
**Fig. 1:** Bland-Altman plots of the results obtained by comparison of complete 24hr urine and fractional urine before centrifugation.

**Figure 1a.** Agreement between C 24 h b and F 1b  
Limits of agreement (Reference Range for difference): -0.101 to 0.076 g/dL  
Meandifference: -0.013 g/dL (CI -0.023 to -0.003)

Range: 0.006 to 0.731 g/dL

**Figure 1b.** Agreement between C 24 h b and F 2b  
Limits of agreement (Reference Range for difference): -0.102 to 0.094 g/dL  
Meandifference: -0.004 g/dL (CI -0.015 to 0.007)

Range: 0.006 to 0.725 g/dL



**Fig. 2:** Bland-Altman plots of the results obtained by comparison of complete 24hr urine and fractional urine after centrifugation.

**Figure 2a.** Agreement between C 24 h a and F 1a  
Limits of agreement (Reference Range for difference): -0.093 to 0.078 g/dL  
Meandifference: -0.008g/dL (CI -0.017 to 0.002)

Range: 0.006 to 0.742g/dL

**Figure 2b.** Agreement between C 24 h a and F 2a  
Limits of agreement (Reference Range for difference): -0.120 to 0.133 g/dL  
Meandifference: 0.006 g/dL (CI -0.008 to 0.020)

Range: 0.006 to 0.749g/dL

### 4. Discussion

We undertook this study to improvise the 24hr urine collection method so that it can be less cumbersome and tedious process. In an attempt to make the urine collection patient friendly, it was decided to take representative sample during each void over 24hr period and evaluate whether parameters measured in the fractional sample would be similar quantitatively to conventional urine collection method. If our hypothesis were to be proved, then, it would give an opportunity to explore new, compact collection

**Table 1:** Demographics of the patient

Particulars	
Mean Age(in years)	42+/- 15
Sex(male in %)	56
No. of apparently healthy volunteers with protein excretion < 0.3g/day (group 1)	31
No. of CKD subjects with protein excretion >0.3g/dL	45
Group 2 : subjects with protein excretion between 0.3 and 1.0 g/day	12
Group 3: Subjects with protein excretion greater than 1.0 g/day	33
Cause ofCKD (n=45)	
Diabetic nephropathy	20
Chronic glomerulonephritis	10
Hypertensive nephropathy	5
Lupus nephritis	5
Nephrotic syndrome	5
Mean Urine volume (mL)	1943 ± 948
MAP (mm Hg)	104±22.1
Serum creatinine (mg/dl)	1.9±0.72
Blood urea (mg/dl)	39.6±26.6
eGFR (ml/min/1.73 m2)	40.21±20.8
Serum albumin (g/dl)	3.64±0.66
Hemoglobin (g/dl)	11.1±2.0

**Table 2:** Details of protein values (g/dL) fractional urine collection and conventional method in various groups

Group no. (no. of subjects)		Conventional urine collection method		Fractional urine collection without preservative (thymol)		Fractional urine Collection with preservative (thymol)	
		C-24 h a	C-24 h b	F1 a	F1 b	F2 a	F2 b
1 (n=31)	Mean	0.007	0.010	0.007	0.008	0.007	0.009
	S.D	0.004	0.014	0.003	0.004	0.003	0.008
	C.I. of mean	0.006-0.009	0.004-0.016	0.006-0.009	0.007-0.010	0.006-0.008	0.005-0.012
2 (n=12)	Mean	0.039	0.040	0.054	0.051	0.046	0.046
	S.D	0.016	0.019	0.028	0.028	0.028	0.029
	C.I. of mean	0.028-0.050	0.027-0.053	0.035-0.073	0.031-0.070	0.026-0.065	0.027-0.066
3 (n=33)	Mean	0.155	0.147	0.164	0.159	0.115	0.144
	S.D	0.161	0.140	0.149	0.142	0.074	0.139
	C.I. of mean	0.071-0.245	0.074-0.212	0.086-0.227	0.084-0.217	0.077-0.113	0.071-0.212

SD – standard deviation, CI – confidence intervals, C-24ha – 24h urine after centrifugation, C-24hb – 24h urine before centrifugation, F1a and F2a – fractional urine collection over 24 h period after centrifugation, F1b and F2b - fractional urine collection over 24 h period before centrifugation

device (Automated device) and further field trial. With fractional collection, through compact automated device one can eliminate the requirement to carry bulky urine containers and enable the subjects to collect urine as per their convenient days and place.

Studies have reported difficulties associated with 24 h urine collection<sup>1</sup> though it is a planned, timed procedure and often opted on a weekend or a holiday since it involves collection in a bulky container. Conventional collection method restricts patient activity or social life on the day of collection. Undeniably, it's a tedious, cumbersome process, handling large volume of sample with storage under temperature controlled conditions, added to the reasons for patients refusal of the conventional sampling

method.

To address this shortcoming of 24h sampling, spot urine samples showed to be as accurate as timed urine collections (complete collection of urine in large containers over fixed time period) for many variables including calculations of variety of indicators of kidney function.<sup>2,3</sup> This compromise of timed sampling is not applicable for some clinical settings and 24h urine collection is still mandatory. Clinical settings where patients have unusual body habitus including obesity and amputations, for those who need chemotherapy regimens, and during evaluations of potential kidney donors, timed urine collections are still standard method of urine collection and evaluation.<sup>4</sup> Rather than using spot sampling or timed urine collection, we

**Table 3:** Comparison of protein values (g/dL) by Bland Altman analysis for various groups

Group no. (no. of subjects)	Details of comparison	Mean difference (g/dL)	Confidence Interval of Mean difference (g/dL)		Limits of agreement (g/dL)	Range (g/dL)
			Lower bound	Upper bound		
<b>1 (n=31)</b>	C-24 h a vs C-24 h b	-0.003	-0.009	0.001	-0.031 to 0.023	0.006 to 0.105
	C-24 h a vs F1 a	0.0002	-0.001	0.002	-0.007 to 0.007	0.006 to 0.066
	C-24 h a vs F2 a	0.0004	-0.001	0.003	-0.008 to 0.011	0.006 to 0.062
	C-24 h b vs F1 b	0.002	-0.002	0.010	-0.027 to 0.035	0.006 to 0.100
<b>2 (n=12)</b>	C-24 h b vs F2 b	0.0016	-0.002	0.010	-0.027 to 0.035	0.006 to 0.094
	C-24 h a vs C-24 h b	-0.002	-0.004	0.008	-0.016 to 0.021	0.011 to 0.074
	C-24 h a vs F1 a	-0.015	-0.023	0.006	-0.048 to 0.026	0.023 to 0.082
	C-24 h a vs F2 a	-0.007	-0.017	0.012	-0.048 to 0.044	0.022 to 0.078
<b>3 (n=33)</b>	C-24 h b vs F1 b	-0.010	-0.023	0.001	-0.048 to 0.025	-0.012 to 0.084
	C-24 h b vs F2 b	-0.006	-0.015	0.019	-0.051 to 0.055	-0.015 to 0.019
	C-24 h a vs C-24 h b	0.008	-0.003	0.029	-0.076 to 0.102	0.028 to 0.738
	C-24 h a vs F1 a	-0.009	-0.038	0.002	-0.132 to 0.095	0.029 to 0.742
	C-24 h a vs F2 a	0.040	-0.025	0.040	-0.174 to 0.190	0.024 to 0.749
	C-24 h b vs F1 b	-0.011	-0.051	-0.013	-0.137 to 0.073	0.014 to 0.731
	C-24 h b vs F2 b	0.003	-0.038	0.005	-0.140 to 0.107	0.010 to 0.725

C-24ha – 24h urine after centrifugation, C-24hb – 24h urine before centrifugation, F1a and F2a – fractional urine collection over 24 h period after centrifugation, F1b and F2b - fractional urine collection over 24 h period before centrifugation

**Table 4:** Comparison of protein values (g/dL) by Bland-Altman analysis in fractional urine for effect of centrifugation and preservative

Group no. (no. of subjects)	Details of comparison	Mean difference (g/dL)	Confidence Interval of Mean difference (g/dL)		Limits of agreement (g/dL)	Range (g/dL)
			Lower bound	Upper bound		
<b>1 (n=31)</b>	F1 a vs F1 b	-0.001	-0.002	0.001	-0.005 to 0.004	0.006 to 0.063
	F2 a vs F2 b	-0.002	-0.003	0.001	-0.012 to 0.009	0.006 to 0.052
	F1 a vs F2 a	0.001	0.0001	0.002	-0.004 to 0.006	0.006 to 0.055
	F1 b vs F2 b	-0.001	-0.002	0.002	-0.010 to 0.012	0.006 to 0.060
<b>2 (n=12)</b>	F1 a vs F1 b	0.003	-0.003	0.007	-0.013 to 0.017	0.024 to 0.094
	F2 a vs F2 b	-0.001	-0.004	0.017	-0.026 to 0.039	0.016 to 0.090
	F1 a vs F2 a	0.009	0.001	0.017	-0.017 to 0.034	0.025 to 0.092
	F1 b vs F2 b	0.004	0.002	0.024	-0.022 to 0.049	0.017 to 0.093
<b>3 (n=33)</b>	F1 a vs F1 b	0.005	-0.016	0.014	-0.087 to 0.085	0.015 to 0.735
	F2 a vs F2 b	-0.029	-0.032	0.009	-0.126 to 0.103	0.006 to 0.736
	F1 a vs F2 a	0.049	-0.004	0.056	-0.144 to 0.197	0.011 to 0.745
	F1 b vs F2 b	0.014	-0.001	0.032	-0.075 to 0.106	0.010 to 0.726

C-24ha – 24h urine after centrifugation, C-24hb – 24h urine before centrifugation, F1a and F2a – fractional urine collection over 24 h period after centrifugation, F1b and F2b - fractional urine collection over 24 h period before centrifugation.

decided to explore a novel approach that meaningfully ease the process of 24h sampling but also maintains its accuracy.

To make it more patient friendly, we decided to look at options of collecting small amounts of urine at the time of voiding. These small amounts collected over a period of 24h could be used for further analysis of patient's biochemical parameters. This study was undertaken to determine whether fractional collection of urine over a period of 24h could give similar biochemical picture as traditional 24h collection.

We have categorized our study population based on a report by Joseph et al.<sup>12</sup> It is generally thought that an excretion rate of more than 300 mg/day constitutes a significant increase in protein excretion though normal excretion is defined as 150–200 mg/day. We have chosen 300 mg/day as a cut-off for apparently healthy patients based on the study by Joseph et al. Their study proposes that different cutoffs should be used in different clinical settings, e.g., a higher value in patients with preexisting renal dysfunction.<sup>12</sup>

In our study, we wanted to evaluate the protein values in conventional and fractional collection method in the background of centrifugation and use of preservative. Firstly, we evaluated the effect of centrifugation in the conventional method. We found that centrifuged samples of conventional urine collection method showed higher protein values by 0.008 g/dL (8 mg/dL) in patients who excreted more than 1.0 g/day. This is in contrast to group 1 and 2 patients whose urine protein values were 3 mg/dL and 2 mg/dL lower in centrifuged samples. This difference is deemed to be clinically insignificant. Urine samples are centrifuged with an intent to eliminate turbidity due to cells, crystals and urinary casts that could give false positive results. HaticeSürer et al.,<sup>13</sup> have studied the effect of centrifugation on three urine protein assays namely benzethonium chloride, benzalkonium chloride and pyragallol red. They reported protein values to be significantly lower after centrifugation and more specifically in samples with low protein concentration. In their study, the protein variation due to centrifugation was more pronounced with benzethonium chloride and benzalkonium chloride methods than pyragallol red method. Their study population had protein ranges much lower in comparison to our study though their results are comparable only in the low protein concentration.

Secondly, we evaluated effect of centrifugation between the methods of collection. We did not find any difference in protein values between centrifuged samples of conventional method versus fractional collection, especially, in group 1. However, in uncentrifuged samples, protein values were higher in conventional collection method by 2 mg/dL in comparison to fractional collection method. The preservative usage did not alter the protein values between conventional and fractional collection method.

In group 2, centrifuged samples of conventional method showed protein values lower than fractional collection by 15 mg/dL (without preservative) in comparison to 9 mg/dL as seen in fractional collection method with preservative. The uncentrifuged samples of conventional method showed protein values lower by 10 mg/dL and 6 mg/dL for urine without and with preservative respectively. In group 3, centrifuged samples of conventional method showed 9 mg/dL lower than fractional method in urine without preservative. However, this was in contrast to samples with preservative showed a difference of 40 mg/dL higher in conventional urine collection method. In uncentrifuged samples of conventional collection method, protein values are lower in sample without preservative in contrast to usage of preservative.

We compared the fractional urine for use of preservative (Thymol). The protein values obtained without thymol is higher by 0.009 to 0.026 g/dL (9 to 26 mg/dL). Thymol seems to interfere with protein estimation. Thymol inhibits bacterial and yeast growth and probably interfere with protein precipitation tests. In higher concentrations, it can precipitate as crystals.<sup>14–16</sup>

24h urine protein estimation is most often used as a screening tool for patients suspected of proteinuria. In a population with either low or high protein excretion, fractional method can be adopted instead of 24h conventional method, since, the difference observed is clinically insignificant.

Ideally, centrifugation is advocated for urine sample to eliminate turbidity. Our study has demonstrated that centrifugation plays a significant role in urine analysis for patients suspected of high protein excretion.

There are several studies that have attempted to compare shorter timed samples with 24h collection. We did extensive literature search to compare and evaluate with previous similar studies but there seemed to be none. Infact, there are studies which compare the spot or random urine and 24h urine protein estimation.<sup>17</sup>

Rather than using spot sampling, our study came up with a novel approach that meaningfully ease the process of 24-hour sampling but also maintains its accuracy. Our results have showed that with proper sampling the volume, urine protein can be measured by our proposed method since there is a good agreement with the conventional technique.

#### 4.1. Limitations of the study

Sample size declined due to non-adherence of the study population to protocol and also due to incomplete urine collection.

Incomplete urine collection was assessed based on urine creatinine excretion instead of PABA.

Representative sample collected by patients that was transferred to small container was assumed to be uniform amongst all the study subjects. However, this aspect would

be difficult to verify.

#### 4.2. Acknowledgement and funding source

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#### 5. Sources of support

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#### 6. Conflict of interest

None.

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