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Citation	Cytotechnology, 68(1), 25-32 <a href="https://doi.org/10.1007/s10616-014-9766-4">https://doi.org/10.1007/s10616-014-9766-4</a>
Issue Date	2016-01
Doc URL	<a href="http://hdl.handle.net/2115/63946">http://hdl.handle.net/2115/63946</a>
Rights	The final publication is available at Springer via <a href="http://dx.doi.org/10.1007/s10616-014-9766-4">http://dx.doi.org/10.1007/s10616-014-9766-4</a>
Type	article (author version)
File Information	Manuscript_revised_final.pdf



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1 **Title**

2 An improved method for western blotting when extracting proteins from mammalian cells  
3 cultured on a collagen gel under serum-free conditions

4

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21

1 **Abstract**

2 Western blotting is a widely used method for detection and quantification of specific proteins  
3 extracted from mammalian cells. In the conventional method of protein extraction, we found  
4 that collagen-containing gels interfered with detection of the p65 protein (one of the subunits in  
5 the NF- $\kappa$ B family of proteins) in human lung adenocarcinoma A549 cells cultured on a collagen  
6 gel containing serum. In contrast, the collagen gels did not affect detection of the GAPDH  
7 protein. Then, we established an improved method for preparation of protein extracts (using  
8 trichloroacetic acid fixation and collagenase treatment) from the cells cultured on the collagen  
9 gel. Using the improved method, we were able to detect p65 proteins without a loss in A549  
10 cells cultured on a collagen gel under serum-free conditions, but we could not if serum was  
11 present in cell culture. Thus, using western blotting and serum-free culture conditions, we  
12 succeeded in comparing the p65 expression between the cells grown in a plastic dish and cells  
13 grown on a collagen gel.

14

15 **Keywords**

16 western blotting; collagen gel; serum; trichloroacetic acid; collagenase; p65

17

## 1 **Introduction**

2 Western blotting (WB) is one of the well-established methods for detecting and quantifying  
3 specific proteins (Laemmli 1970; Towbin et al. 1979). A recent study reported that substrate  
4 stiffness affects cell behavior. For example, the rigidity of a collagen matrix regulates collective  
5 motility and the expression of adhesion proteins (Wang et al. 2003; Haga et al. 2005).  
6 Furthermore, stiffer substrates may enhance cancer progression (Paszek et al. 2005, Levental et  
7 al. 2009). Accordingly, quantitative analysis of specific proteins in the cells cultured on stiff and  
8 soft substrates is gaining wider interest. In particular, the proteins that regulate intracellular  
9 biochemical signaling affected by substrate stiffness have been analyzed. NF- $\kappa$ B is one of the  
10 regulators of such signaling and plays important roles in inflammatory reactions and cancer  
11 progression (Karin et al. 2002; Li and Verma 2002; Ishihara et al. 2013). We focused on the p65  
12 protein, one of the NF- $\kappa$ B subunits.

13 In this study, we found that collagen-containing gels hamper detection of the p65 protein  
14 in the cells cultured on a collagen gel containing serum. Then, we established an improved  
15 method for western blotting using trichloroacetic acid (TCA) fixation and collagenase treatment  
16 to extract proteins from mammalian cells cultured on a collagen gel under serum-free conditions.  
17 TCA is used as a fixative of cells or tissues for various experiments, such as western blotting  
18 and histochemical analysis (Cafruny 1957; Musial and Eissa 2001). Although the mechanism  
19 has not been identified yet, TCA fixation is thought to be caused by reactions between the  
20 negatively charged chloroacetate groups of TCA and positively charged amino groups of the  
21 proteins. Collagenases are enzymes that hydrolyze the triple helical region of collagens (Bond  
22 and Van Wart 1984). Thus, by means of the collagenase treatment, we were able to dissolve the  
23 collagen gel and remove it from cell lysates. In previous studies, researchers often lysed the  
24 cells without trichloroacetic acid and collagenase treatment for western blotting (Wang et al.

1 2003; Engler et al. 2006; Tsutsumi et al. 2009; Wu et al. 2009). We refer to this approach as “the  
2 conventional method” in this study. Compared to the conventional method, our improved  
3 method allowed for detection of the band of p65 proteins without losses.

4

1 **Materials and methods**

2 **Cell culture and material preparation**

3 The A549 human lung adenocarcinoma cell line (American Type Culture Collection, Manassas,  
4 VA), HT1080 human fibrosarcoma cell line (Riken Cell Bank, Tsukuba, Japan), and NIH-3T3  
5 mouse fibroblast cell line (Riken Cell Bank) were cultured in Dulbecco's Modified Eagle  
6 Medium (DMEM; Sigma, St Louis, MO) containing 10% fetal bovine serum (BIST TECH,  
7 Equitech Bio Inc., Kerrville, TX or Biowest, Nuaille, France) and 1% antibiotic/antimycotic  
8 solution (Sigma). Mouse primary fibroblasts were isolated from the abdominal subcutaneous  
9 tissue of a male Slc:ICR retired mouse (Japan SLC, Inc., Hamamatsu, Japan). The animal  
10 experiment was strictly compliant with the animal care guidelines of Hokkaido University. The  
11 cells were incubated at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Each cell type was  
12 passaged and used for experiments unless the cells showed aberrant morphology. For western  
13 blotting, 10<sup>5</sup> cells were seeded on a 35 mm plastic dish (>1 GPa) coated with type I collagen  
14 (Cell matrix I-C; Nitta Gelatin, Osaka, Japan) or on a 500 μL of type I collagen gel (1.6 mg/mL,  
15 approximately 0.6 kPa; Cell matrix I-P, Nitta Gelatin) in a 35 mm plastic dish. The cells were  
16 cultured for 24 h for western blotting. For serum-free culture, we seeded 10<sup>5</sup> cells in a culture  
17 dish filled with serum-free DMEM and cultured them for 24 h. To add a collagen gel to a cell  
18 lysate, we prepared 500 μL of type I collagen gel (1.6 mg/mL, approximately 0.6 kPa; Cell  
19 matrix I-P, Nitta Gelatin) in a 35-mm plastic dish. The gel was covered with serum-containing  
20 or serum-free DMEM and incubated at 37 °C in a humidified incubator with 5% CO<sub>2</sub> for 24 h.

21

22 **Antibodies**

23 For western blotting, we used the following antibodies: an anti-NF-κB p65 antibody (IBL,  
24 Fujioka, Japan; cat. # 18667), anti-GAPDH (Ambion, Foster City, CA; cat. # 1103016),

1 anti-integrin  $\beta$ 1 (BD BioScience, San Jose, CA; cat. # 610467), anti-cyclin E1 (Cell Signaling  
2 Tech, Danvers, MA; cat. # 4129), HRP-conjugated anti-rabbit IgG (Cell Signaling Tech; cat. #  
3 7074), and an HRP-conjugated anti-mouse IgG antibody (Bio-Rad, Hercules, CA; cat. #  
4 70-6516).

5

## 6 **Western blotting**

7 In the conventional method for extraction of proteins from the cells cultured in a plastic dish  
8 and on a collagen gel, we removed the medium from the cell sample and lysed the cells with  
9 200  $\mu$ L of SDS sample buffer (0.25 M Tris-HCl pH 8.8, 5% dithiothreitol, 2.3% SDS, 10%  
10 glycerol, 0.01% bromophenol blue). This SDS sample buffer was used for lysing the whole-cell  
11 extract. After that, the cell lysate was homogenized, sonicated using an ultrasonic homogenizer  
12 (UH-50; SMT Company, Tokyo, Japan), and then boiled at 95 °C for 5 min.

13 In the improved method for extraction of proteins from the cells cultured in a plastic dish,  
14 we removed the media from the cell sample and added 500  $\mu$ L of 10% trichloroacetic acid (TCA,  
15 Sigma) in phosphate-buffered saline (PBS). The sample was incubated for 3 min on ice and  
16 washed 3 times with 1 mL of PBS on ice. We lysed the cells with 200  $\mu$ L of SDS sample buffer.  
17 After that, the cell lysate was homogenized, sonicated using the ultrasonic homogenizer (UH-50,  
18 SMT company, Tokyo, Japan), and then boiled at 95 °C for 5 min.

19 In the improved method for extraction of proteins from the cells cultured on a collagen  
20 gel, we removed the medium from the cell sample and added 500  $\mu$ L of 10% TCA in PBS. Then,  
21 the sample was incubated for 3 min on ice and washed 3 times with 1 mL of PBS on ice. Next,  
22 we added 250  $\mu$ L (half a volume of the collagen gel) of 0.1% collagenase-L (Nitta Gelatin) in  
23 PBS to the sample and incubated the mixture at 37 °C for 1 h to allow for digestion of the  
24 collagen gel. The cells were collected into microtubes and centrifuged at 14,000 revolutions per

1 minute (17,700 g) for 3 min at 4 °C. After removing the supernatant, we lysed the cells with 200  
2 μL of SDS sample buffer. After that, the cell lysate was homogenized, sonicated using the  
3 ultrasonic homogenizer (UH-50, SMT company, Tokyo, Japan), and then boiled at 95 °C for 5  
4 min.

5 We used 10% polyacrylamide gels for SDS-PAGE (20 mA per gel, 1 h). We applied the  
6 same volume (4 μL as usual) of cell lysates to wells of the polyacrylamide gel in order to  
7 compare the protein expression among the cell lysates. After SDS-PAGE, blotting to  
8 polyvinylidene difluoride membranes was performed (92 mA per gel, 30 min for p65 and  
9 GAPDH, 60 min for integrin β1 and cyclin E1). After the blotting, the membranes were  
10 incubated in 5% skim milk in Tris-buffered saline plus Tween 20 (TBS-T) for 1 h at room  
11 temperature. The membranes were incubated with primary antibodies (p65 1:1,000, GAPDH  
12 1:1,000,000–1:10,000,000, integrin β1 1:10,000, and cyclin E1 1:100,000) for 1 h at room  
13 temperature and/or overnight at 4 °C. After 3 washes with TBS-T, the membranes were  
14 incubated with secondary antibodies (HRP-conjugated anti-rabbit IgG antibody, 1:10,000  
15 dilution for p65; HRP-conjugated anti-mouse IgG antibody, 1:200,000 dilution, for GAPDH;  
16 and HRP-conjugated anti-mouse IgG antibody, 1:10,000 dilution, for integrin β1 and cyclin E1)  
17 for 1 h at room temperature. Signals were detected with Immobilon Western Chemiluminescent  
18 HRP substrate (Millipore, Billerica, MA). The relative expression of the proteins was assessed  
19 by means of the Image J software (National Institutes of Health, USA). Statistical analysis was  
20 performed using Student's or Welch's *t* test.

21

## 1 **Results and discussion**

2 First, we examined the expression of p65 and GAPDH proteins in the cells on a collagen gel as  
3 a soft substrate and that on a plastic dish as a stiff substrate in the presence of serum, by means  
4 of WB using the conventional method for preparing cell extracts (Fig. 1a). Extracts of the cells  
5 cultured in a plastic dish and on a collagen gel using this method are labeled in the figure as P  
6 and G respectively. GAPDH served as an internal control. The intensity of p65 bands in P was  
7 higher than in G (Fig. 1b). We elucidated whether this difference is due to the different level of  
8 p65 expression when the cells are on different substrates or due to the experimental artifacts  
9 related to the collagen gel. To this end, we added a collagen gel to the cell lysate prepared from  
10 the cells on a plastic dish. The collagen gel was preincubated with serum-containing media to  
11 mimic the collagen gel in cell culture with serum-containing media and to assess the effects of  
12 serum in the gel on the efficiency of protein detection. This condition was abbreviated as P-G  
13 (Fig. 1a). The p65 band of condition P showed higher intensity compared to P-G (Fig. 1b).  
14 These results indicated that collagen gels preincubated with serum-containing media worsened  
15 p65 detection and made it impossible to compare the expression of p65 in the cells on a collagen  
16 gel as a soft substrate with that on a plastic dish as a stiff substrate by means of WB.

17 To avoid the artificial deterioration of p65 detection in the cells cultured on a collagen gel,  
18 we improved the method of cell extraction by introducing trichloroacetic acid (TCA) fixation  
19 and collagenase treatment (Fig. 2a). We tested whether the improved method eliminates the  
20 problems with p65 detection caused by the collagen gel. At the same time, the effect of serum in  
21 a collagen gel on p65 detection was examined. We prepared 3 protein extracts from the cells on  
22 a plastic dish cultured with serum-containing media. We prepared the following samples: P'(+:  
23 protein extraction without a collagen gel, P'-G'(+/+): extraction when a collagen gel was added  
24 along with preincubation with serum-containing media, and P'-G'(+/-): extraction when a

1 collagen gel was added, but preincubation with serum-free media. P'(+) was implemented using  
2 the improved method for protein extraction from the cells cultured on a plastic dish (shown as P'  
3 in Fig. 2a) whereas P'-G'(+/+) and P'-G'(+/-) were implemented using the method for protein  
4 extraction from the cells on a plastic dish with a collagen gel added later (shown as P'-G' in Fig.  
5 2a). The WB results indicated that P'(+) displayed more intense p65 bands than did P'-G'(+/+),  
6 whereas P'(+) showed almost the same intensity of the p65 bands compared to P'-G'(+/-) (Fig.  
7 2b). These results indicated that we enabled detection of p65 bands without losses in the cells  
8 cultured with a serum-free collagen gel when we used the improved method. In contrast, p65  
9 detection was compromised in the cells cultured with a collagen gel preincubated with  
10 serum-containing media when we used the improved method. Coomassie brilliant blue staining  
11 revealed that the P'-G'(+/+) resulted in remarkable intensity of the protein bands, whose  
12 molecular weight was 50–75 kDa, whereas the other approaches did not (Fig. 3). This protein  
13 may be albumin, which is a 66 kDa protein and a major component of serum (Peters 1977).  
14 Thus, albumin may interfere with p65 detection by blocking the reaction between the antibody  
15 and p65 as an antigen.

16 Next, we compared the conventional method with the improved method in the detection  
17 of proteins in A549 cells to assess the possible advantages of the improved method. In both  
18 types of samples (plastic dish and collagen gel), the improved method enhanced the detection of  
19 p65 compared to the conventional method when cells were cultured in the presence of serum  
20 (Fig. 4). Under serum-free conditions, the improved method enhanced the detection of p65 in  
21 the samples from cells grown on a collagen gel (Fig. 4). Furthermore, detection of integrin  $\beta$ 1  
22 and cyclin E1 was also bettered by the improved method in the samples from cells grown on a  
23 collagen gel, except for integrin  $\beta$ 1 in the cells grown under serum-free conditions (Fig. 4).  
24 These results demonstrated that the improved method enhances protein detection in A549 cells

1 grown in a plastic dish in the presence of serum or on a collagen gel either with or without  
2 serum.

3 For further confirmation of the advantage of the improved method, we attempted to detect  
4 p65 and GAPDH in different batches of A549 cells using the conventional or the improved  
5 method. We prepared cell extracts from HT1080 human fibrosarcoma cells, NIH-3T3 mouse  
6 fibroblast cells, and mouse primary fibroblasts. Just like in A549 cells, p65 detection was  
7 enhanced by the improved method in those other cell lines when they were cultured in a plastic  
8 dish and on a collagen gel in the presence of serum (Fig. 5). Under serum-free conditions,  
9 because NIH-3T3 cells and mouse primary fibroblasts could barely adhere to a plastic dish and  
10 a collagen gel, we could not detect distinctive bands of p65 and GAPDH. Overall, in several  
11 types of cells, the improved method resulted in more visible bands of p65 compared to the  
12 conventional method.

13 Our results suggest that the detection of p65 is impeded by a collagen gel and serum. Thus,  
14 the p65 bands—from the A549 and HT1080 cells grown on a collagen gel under serum-free  
15 conditions—were more visible after the improved method, which can exclude a collagen gel  
16 from lysates (Fig. 4 and 5). Therefore, a collagen gel impedes the detection of p65 in western  
17 blotting. In addition, we prepared lysates of A549 and HT1080 cells grown in a plastic dish in  
18 the presence of serum using the conventional method (which cannot exclude serum proteins  
19 from lysates; Fig. 6). This approach resulted in weaker bands of p65 compared to lysates of the  
20 cells grown in a plastic dish under serum-free conditions (Fig. 4 and 5). These results suggest  
21 that serum hampers p65 detection. Thus, removal of collagen gel and serum is important for  
22 sensitive detection of p65 proteins (without losses).

23 In contrast to p65 detection, GAPDH detection was almost the same for the lysates  
24 prepared according to the conventional and the improved method, in each culture condition.

1 This may be because the collagen gel and serum affect protein detection depending on the  
2 properties of a protein under study, such as molecular weight. Indeed, molecular weight of  
3 GAPDH (approximately 37 kDa) is substantially less than that of p65 (approximately 65 kDa).  
4 Thus, detection of a protein may be affected by a collagen gel and serum, depending on the  
5 protein's molecular weight.

6 Next, we examined the necessity of TCA fixation and collagenase treatment in the  
7 improved method for the lossless protein detection in the cells cultured on a collagen gel under  
8 serum-free conditions. We compared the following samples: a sample treated with collagenase  
9 after TCA fixation to a sample without TCA fixation and collagenase treatment, a sample treated  
10 only with TCA, and a sample treated only with collagenase. The former showed stronger  
11 intensity of the p65 bands, although the differences were not significant (Fig. 7). Thus, both  
12 TCA fixation and collagenase treatment were necessary for the protein detection without losses  
13 in the improved method.

14 Finally, using the improved method, we compared the expression of the p65 protein in  
15 A549 cells cultured with serum-free media on a plastic dish with that on a collagen gel. The  
16 expression level of p65 protein was almost the same (Fig. 8). This result suggested that the  
17 difference in substrate stiffness does not affect the expression of the p65 protein in A549 cells in  
18 serum-free culture.

19 We showed here that the method including TCA fixation and collagenase treatment  
20 improved protein detection. In previous studies, conventional cell lysis methods, which do not  
21 involve TCA fixation and collagenase treatment, were often used for western blotting (Engler et  
22 al. 2006; Tsutsumi et al. 2009; Wu et al. 2009). In these studies, the cells were cultured on  
23 polyacrylamide gels, plastic plates, or dishes. Under these culture conditions, conventional  
24 methods may not be particularly problematic because there is almost no effect of collagen gels

1 on protein detection. In contrast, in our experiments, including cell culture on a collagen gel, the  
2 improved method is essential for sensitive detection of specific proteins such as p65, integrin  $\beta$ 1,  
3 and cyclin E1 without losses caused by collagens. In western blotting analysis, our improved  
4 method is a powerful way to detect proteins in cells cultured with a collagen gel.  
5

1 **Acknowledgements**

2 This study was supported by Grants-in-Aid for Scientific Research (B) (24390285), Scientific  
3 Research (C) (26430104) and Scientific Research in Innovative Areas (25127701) to H.H.,  
4 Young Scientists (B) (23770167) and Scientific Research in Innovative Areas (24106502) to  
5 T.M., Scientific Research (B) (25287106) to K.K. from the Japanese Ministry of Education,  
6 Culture, Sports, Science, and Technology, Young Scientists (B) (10719933) to S.I. The authors  
7 thank Sawa Kobayashi for technical assistance.

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17

1 **Figure Captions**

2 **Fig. 1**

3 In the conventional method, a collagen gel interferes with detection of the p65 protein in human  
4 lung adenocarcinoma A549 cells cultured on a serum-containing collagen gel. (a) A  
5 conventional procedure of preparing the protein extracts from the cells cultured on a plastic dish  
6 or on a collagen gel. The cells were cultured with serum-containing media. P: protein extraction  
7 from the cells cultured on a plastic dish. G: extraction from the cells cultured on a collagen gel.  
8 P-G: extraction from the cells cultured on a plastic dish: a collagen gel was added to the  
9 extraction to assess the artificial deterioration of the protein detection because of the collagen  
10 gel. In the P-G method, the collagen gel was preincubated with a serum-containing medium to  
11 evaluate the effect of serum in the gel on the protein detection. (b) Western blotting results for  
12 p65 and GAPDH in the samples prepared using the conventional procedure shown in (a). Serum  
13 in cell culture (+): the cells were cultured with a serum-containing medium. Serum in added gel  
14 (+): a P-G sample means that a collagen gel was added along with preincubation with a  
15 serum-containing medium. The graph shows relative intensity of p65 bands from 3 independent  
16 experiments. The data are presented as mean  $\pm$  SE; \* $p < 0.05$  according to Welch's  $t$  test

1 **Fig. 2**

2 An improved method allows us to detect the p65 protein without losses during protein  
3 extraction from lung adenocarcinoma A549 cells cultured on a collagen gel under serum-free  
4 conditions. (a) An improved procedure of preparing the extracts from the cells cultured on a  
5 plastic dish or on a collagen gel. The added steps in the improved procedure are highlighted in  
6 red. P': protein extraction from the cells cultured on a plastic dish. G': extraction from the cells  
7 cultured on a collagen gel. P'-G': extraction from the cells cultured on a plastic dish, where a  
8 collagen gel was added to the samples before collagenase treatment. (b) GAPDH and p65 data  
9 from western blotting after the P' and P'-G' methods where the samples were prepared using the  
10 improved procedure shown in (a). Serum in cell culture (+): the cells were cultured with  
11 serum-containing media. Serum in added gel (+): P'-G' means that a collagen gel was added  
12 before collagenase treatment, and the collagen gel was preincubated with a serum-containing  
13 medium. Serum in added gel (-): P'-G' means that a collagen gel was added before collagenase  
14 treatment, and the collagen gel was preincubated with a serum-free medium. The graph shows  
15 the relative intensity of p65 bands from 3 independent experiments. The data are presented as  
16 mean  $\pm$  SE; \* $p < 0.05$  according to Welch's  $t$  test

17

1 **Fig. 3**

2 The P'-G' sample (where we added a collagen gel preincubated with a serum-containing  
3 medium) shows a protein band of remarkable intensity, whose molecular weight was 50–75 kDa,  
4 detected by means of Coomassie brilliant blue staining. The Coomassie brilliant blue staining  
5 results in the P' and P'-G' samples prepared using the improved procedure shown in Fig. 2a. P':  
6 protein extraction from the cells cultured on a plastic dish. P'-G': extraction from the cells  
7 cultured on a plastic dish, where a collagen gel was added to the samples before collagenase  
8 treatment. Serum in cell culture (+): the cells were cultured with serum-containing media.  
9 Serum in added gel (+): the P'-G' sample where we added a collagen gel before collagenase  
10 treatment, and the collagen gel was preincubated with a serum-containing medium. Serum in the  
11 added gel (-): the P'-G' sample where we added a collagen gel before collagenase treatment, and  
12 the collagen gel was preincubated with a serum-free medium. A representative result from 3  
13 independent experiments is shown

1 **Fig. 4**  
2 Detection of p65, integrin  $\beta$ 1, and cyclin E1 is enhanced by the improved method in A549 cells  
3 cultured in a plastic dish and on a collagen gel. The figure shows western blotting data on p65,  
4 integrin  $\beta$ 1, cyclin E1, and GAPDH after protein extraction from the cells cultured in a plastic  
5 dish (P) or on a collagen gel (G). Serum in cell culture (+): the cells were cultured in  
6 serum-containing media. Serum in cell culture (-): the cells were cultured in serum-free media.  
7 Method (C): extraction according to the conventional method shown in Fig. 1a. Method (I):  
8 extraction according to the improved method shown in Fig. 2a. The graph shows relative  
9 intensity of p65, integrin  $\beta$ 1, and cyclin E1 bands from 3 independent experiments. The data are  
10 presented as mean  $\pm$  SE; \* $p < 0.05$  according to Student's or Welch's  $t$  test  
11

1 **Fig. 5**

2 Detection of p65 is enhanced by the improved method in various types of cells cultured in a  
3 plastic dish and on a collagen gel. The figure shows western blotting data on p65 and GAPDH  
4 after protein extraction from the cells cultured in a plastic dish (P) or on a collagen gel (G).  
5 Serum in cell culture (+): the cells were cultured in serum-containing media. Serum in cell  
6 culture (-): the cells were cultured in serum-free media. Method (C): extraction according to the  
7 conventional method shown in Fig. 1a. Method (I): extraction according to the improved  
8 method shown in Fig. 2a. HT: human fibrosarcoma HT1080 cells, 3T3: mouse fibroblast  
9 NIH-3T3 cells, MP: mouse primary fibroblasts. The graph shows relative intensity of p65 bands  
10 in each cell type from 3 independent experiments. The data are presented as mean  $\pm$  SE,  $*p <$   
11 0.05 according to Student's or Welch's  $t$  test

12

1 **Fig. 6**

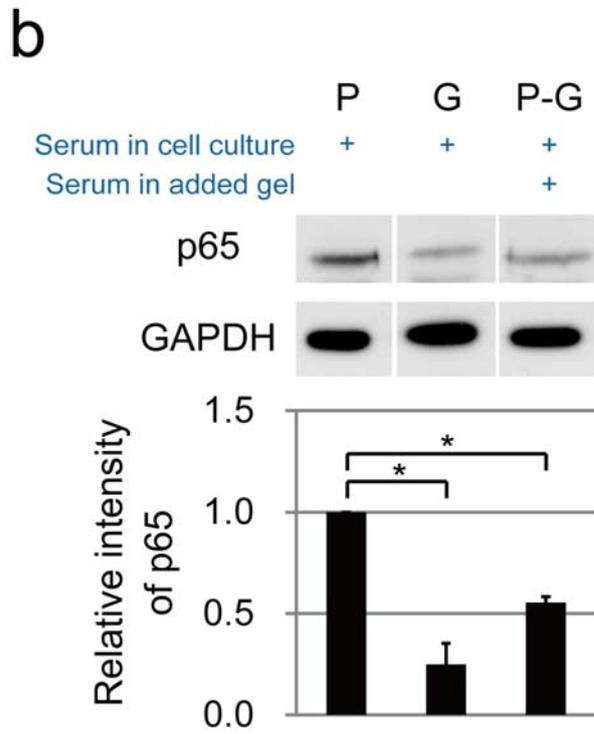
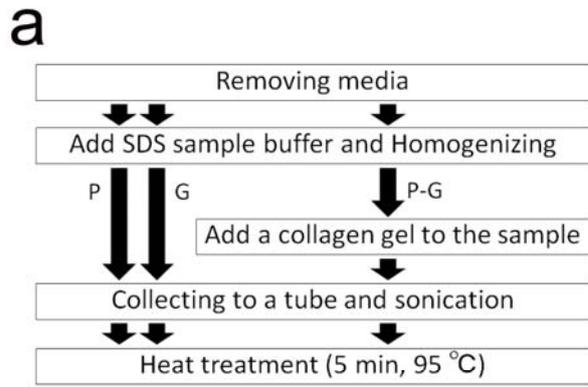
2 The lysate (of A549 cells grown in a plastic dish in the presence of serum) prepared using the  
3 conventional method shows a protein band of remarkable intensity, whose molecular weight  
4 was 50–75 kDa, detected by means of Coomassie brilliant blue staining. The Coomassie  
5 brilliant blue staining results in lysates from the cells cultured in a plastic dish (P). Serum in cell  
6 culture (+): the cells were cultured in serum-containing media. Serum in cell culture (-): the  
7 cells were cultured in serum-free media. Method (C): extraction according to the conventional  
8 method shown in Fig. 1a. Method (I): extraction according to the improved method shown in  
9 Fig. 2a. A representative result from 3 independent experiments is shown

1 **Fig. 7**  
2 TCA fixation and collagenase treatment are necessary for p65 protein detection without losses  
3 in the improved method shown in Fig. 2a. Western blotting data on p65 and GAPDH after  
4 protein extraction from the cells cultured on a collagen gel. Serum in cell culture (-): the cells  
5 were cultured with serum-free media. TCA (+): the cells fixed with 10% TCA and washed 3  
6 times before protein extraction. TCA (-): the cells without TCA fixation and after washes before  
7 extraction. Collagenase (+): the cells treated with 0.1% collagenase after removal of the  
8 collagen gel before protein extraction. Collagenase (-): the cells without collagenase treatment  
9 after removal of the collagen gel before extraction. The graph shows relative intensity of p65  
10 bands from 3 independent experiments. The data are presented as mean  $\pm$  SE

1 **Fig. 8**

2 There are no significant differences in p65 expression between lung adenocarcinoma A549 cells  
3 cultured in a plastic dish and the same cells cultured on a collagen gel under serum-free  
4 conditions. Western blotting data on p65 and GAPDH after protein extraction—according to the  
5 improved method shown in Fig. 2a—from the cells cultured in a plastic dish (P') or on a  
6 collagen gel (G'). Serum in cell culture (-): the cells were cultured in serum-free media. The  
7 graph shows relative intensity of p65 bands from 3 independent experiments. The data are  
8 presented as mean  $\pm$  SE

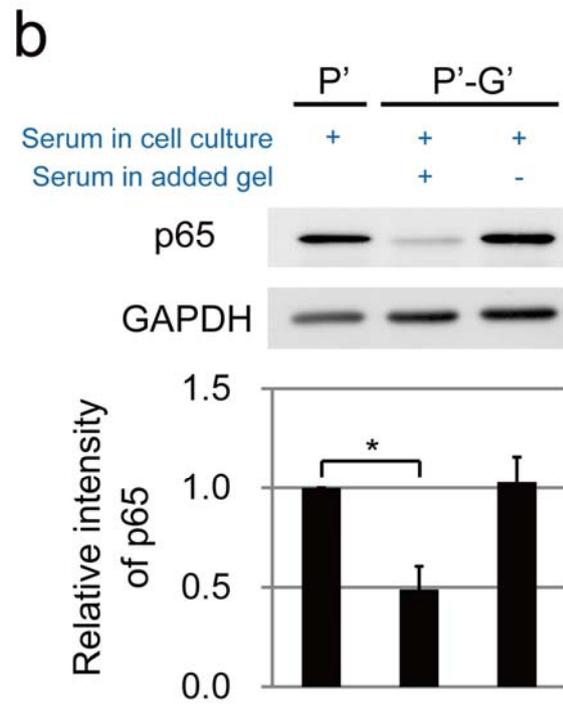
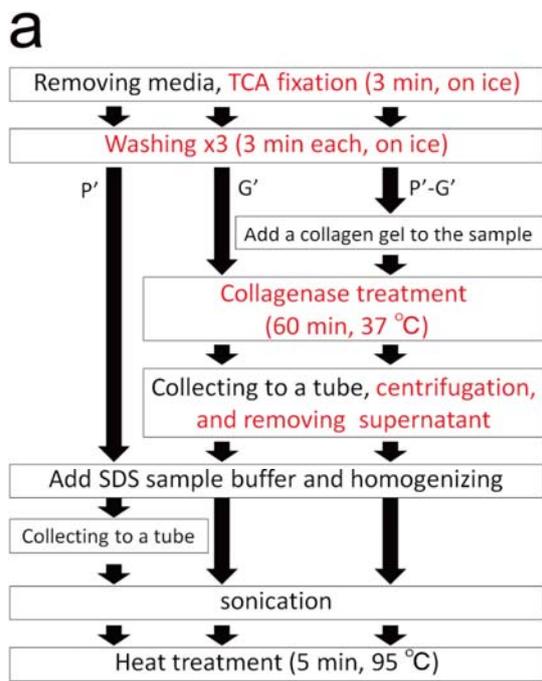
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2 **Fig. 1**

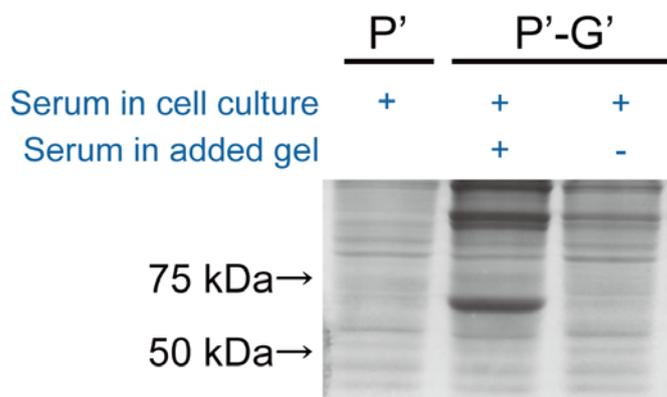
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2 **Fig. 2**

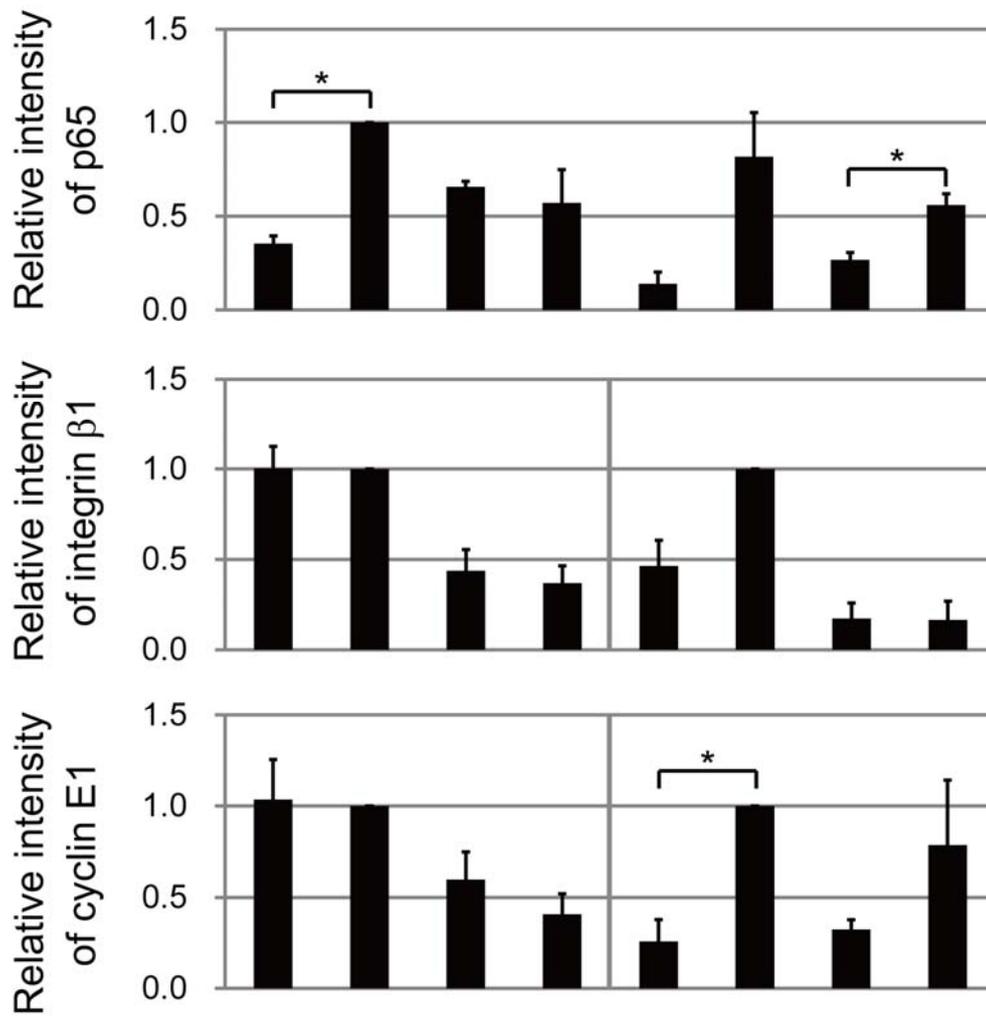
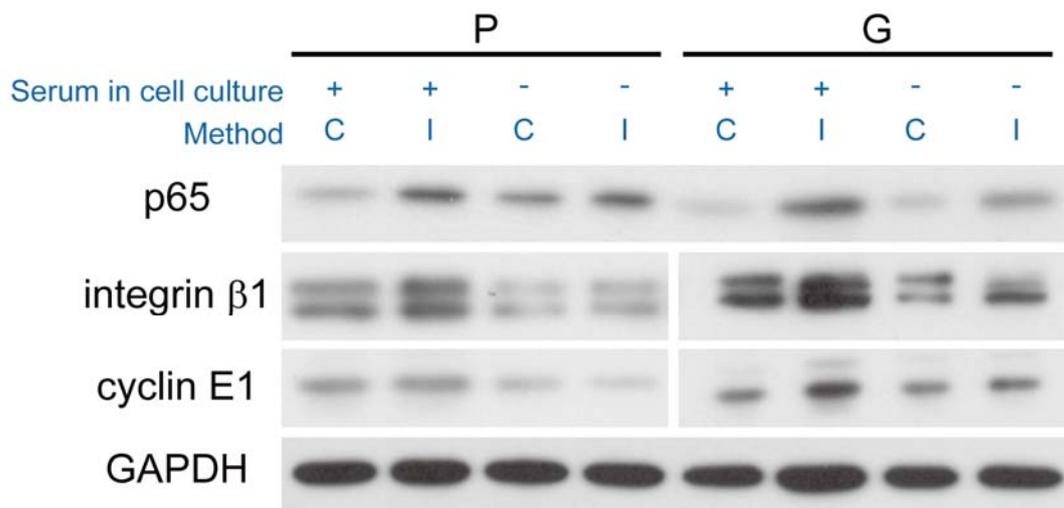
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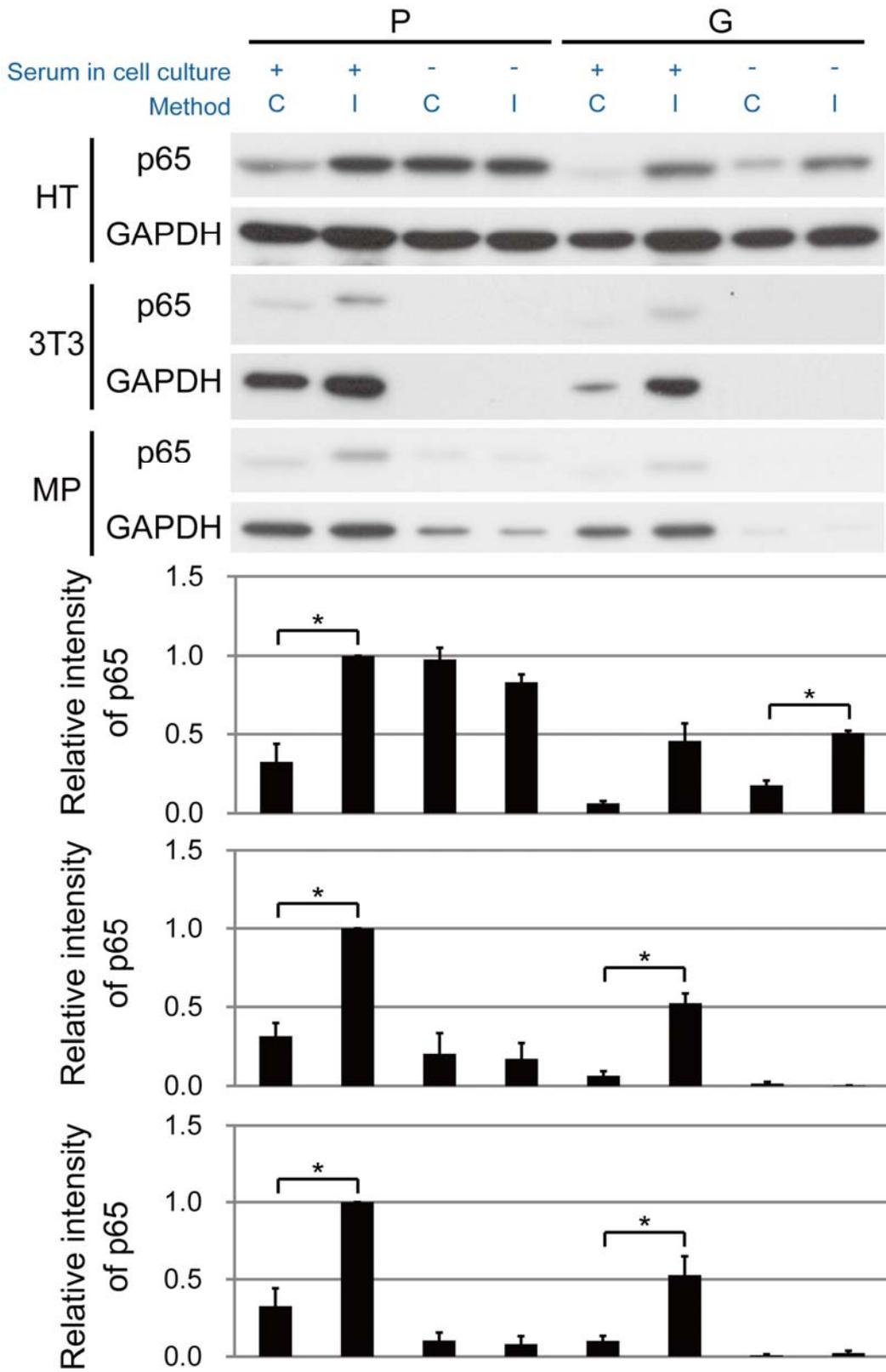
2 **Fig. 3**

3



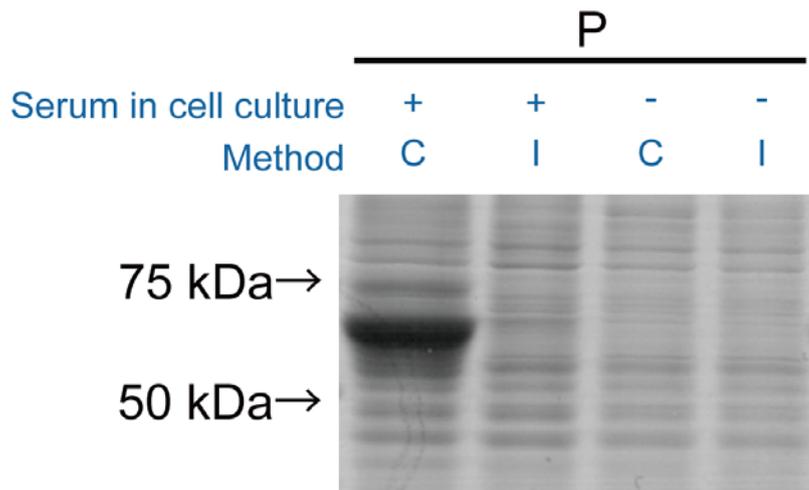
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2 **Fig. 4**



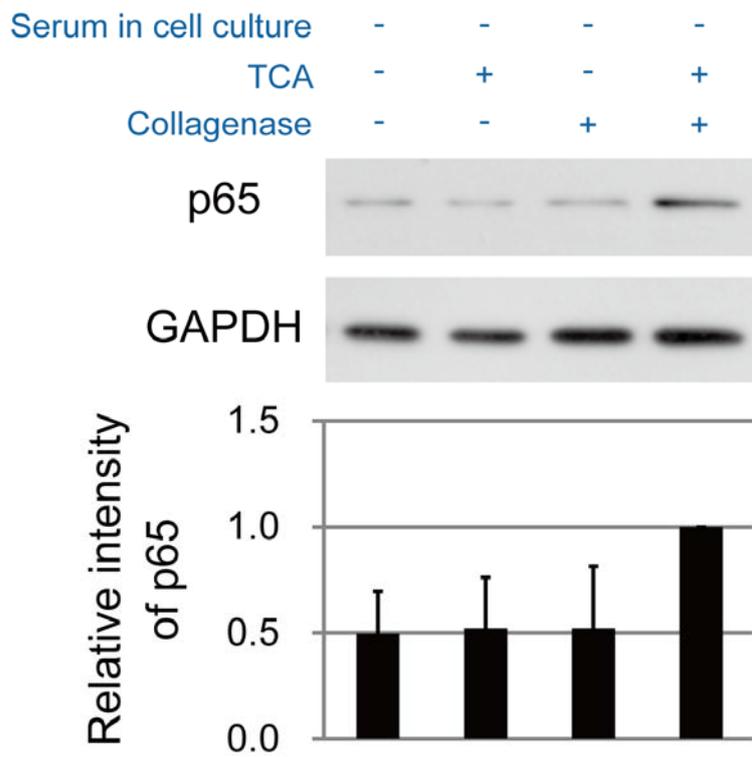
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2 Fig. 5

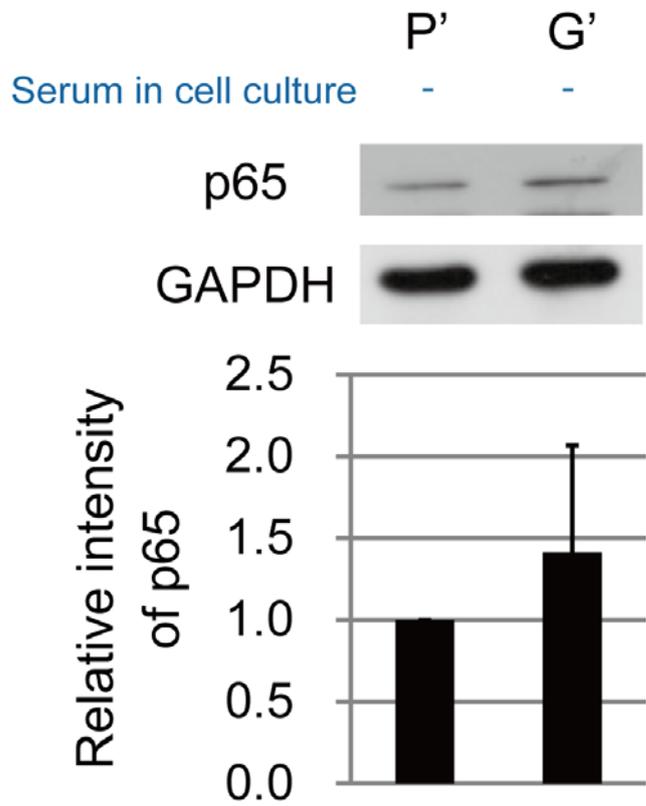


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2 **Fig. 6**



1  
2 **Fig. 7**  
3



1

2 **Fig. 8**