

## Detecting p53 Isoforms at Protein Level

Virginie Marcel\*, Marie P. Khoury\*, Kenneth Fernandes, Alexandra Diot, David P. Lane, and Jean-Christophe Bourdon

### Abstract

The human p53 protein isoforms are expressed in several cell lines and modulate p53 tumor suppressor activity, mainly through modulation of gene expression (1–4). Thus, identifying the pattern of p53 isoforms expression in cell lines is a key step for future studies of the p53 network (5). At the moment, the detection of p53 protein isoforms is based on the use of a panel of antibodies allowing their identification by comparing their molecular weights and their detection pattern by different antibodies (6). Here, classical protocols supplemented with technical know-how are described to detect p53 protein isoforms at protein level by Western blotting and immunoprecipitation. Furthermore, a simple method to study the impact of p53 protein isoforms on p53 transcriptional activity through luciferase reporter gene assays is provided.

**Key words:** p53 protein isoforms, Antibodies, Western blotting, Immunoprecipitation, Luciferase reporter gene assays, Protein detection, p53 tumor suppressor protein, Animal model

---

## 1. Introduction

The human tumor suppressor *TP53* gene expresses several p53 transcripts due to the use of alternative promoters, splicing sites, and/or internal initiation sites of translation (1, 7–9). The different p53 transcripts encode at least 12 protein isoforms produced by the combination of four distinct N-terminal domains (TA,  $\Delta 40$ ,  $\Delta 133$ , and  $\Delta 160$ ) and three distinct C-terminal domains ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) (see Fig. 1) (5). In the N-terminus, the TA forms (initiation site of translation: ATG-1) exhibit a transactivation domain (TAD) and a DNA-binding domain

---

\* Both authors have contributed equally to this work.

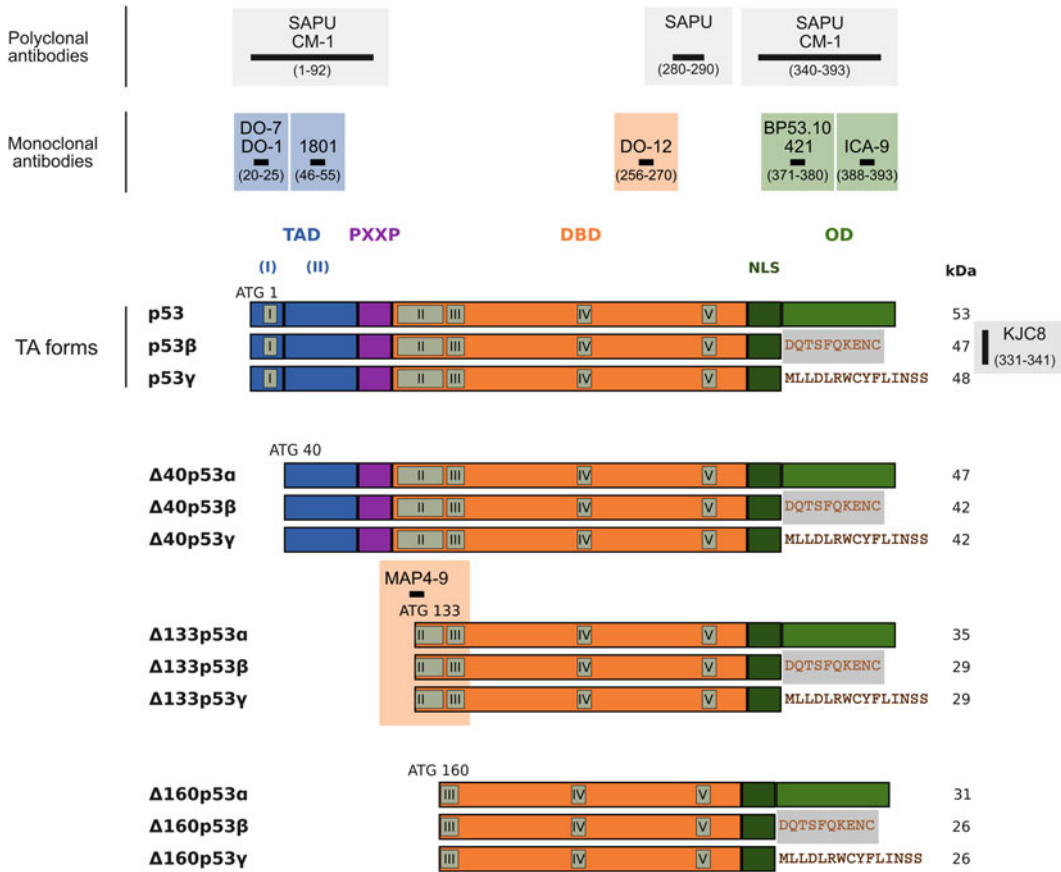


Fig. 1. The human p53 protein isoforms. At least 12 p53 protein isoforms are expressed by the *TP53* gene. Four N-truncated forms (TA, Δ40, Δ133, and Δ160) can be combined to three distinct C-terminal forms (α, β, and γ). As compared to the canonical p53 protein (or TAp53α), the Δ40 forms lack the transactivation domain I (TADI), while the Δ133 and Δ160 forms lack the entire TAD and part of the DNA-binding domain (DBD). In the C-terminus, the α forms contain an oligomerization domain (OD), whereas β and γ forms include novel residues instead of the OD. By Western blotting, the detection of p53 protein isoforms is based on their individual molecular weight (*right panel*) and their distinct recognition pattern by a panel of monoclonal and polyclonal anti-p53 antibodies (*upper panel*). TAD: transactivation domain, *blue*; PXXP: proline-rich domain, *violet*; DBD: DNA-binding domain, *orange*; NLS: nuclear localization signal, *dark green*; OD: oligomerization domain, *light green*; *gray boxes*: conserved domains; ATG: codon used to initiate each N-truncated p53 protein isoform; kDa: kilo Dalton, theoretical protein molecular weight; *colored boxes*: monoclonal and polyclonal anti-p53 antibodies; *numbers within brackets*: epitopes.

(DBD), while the Δ40 forms (ATG-40) lack part of the TAD and the Δ133/Δ160 forms (ATG-133 and ATG-160, respectively) lack the entire TAD and part of the DBD. In the C-terminus, the α forms contain an oligomerization domain (OD), which is replaced by 10 or 15 new residues in β and γ forms, respectively. Among the p53 isoforms, the canonical p53 protein corresponds to the largest protein expressed by *TP53* with a TAD, a DBD, and a C-terminal domain (OD) and thus termed p53α or TAp53α (see Fig. 1). Several studies have reported the interplay between p53 protein isoforms, resulting in the modulation of p53 tumor suppressor activity mainly through regulation of gene expression (2-4, 7).

**Table 1**  
**Panel of commercially and non-commercially available primary antibodies to detect p53 protein isoforms (see Note 9)**

Species	Primary antibody	Source	Western blotting (µg/ml)	Immuno-precipitation (µg)	Immunostaining (µg/ml)	Ex. supplier/catalog number	Ref.
Human	DO-1	Mouse	1	5	1	Santa Cruz Biotechnology/sc-126	(1, 8)
	DO-12	Mouse	5	5	5	Convance/SIG-3520	(1)
	CM1	Rabbit	1	5	1		(1)
	SAPU	Sheep	1	5	1		(1)
	KJC8	Rabbit	1	10	1		(3)
	MAP4-9	Rabbit	3	nd	nd		
<i>Drosophila</i>	anti-Dmp53	Rabbit					(11)
	PolyCt	Rabbit					(12)
Zebrafish	5.1	Mouse					(13)
	9.1	Mouse					(13)

nd not determined, ex. example, ref. reference

The human p53 protein isoforms have been detected at endogenous level in several human cell lines (1, 7, 8). Using the current available antibodies (see Table 1), the detection of p53 isoforms is mainly based on the comparison of signal pattern given by a panel of antibodies, since p53 protein isoforms share a common amino-acid sequence and thus similar epitopes (see Fig. 1). For example, the detection of p53 protein isoforms by Western blotting is based on (1) a different reactivity to a panel of several antibodies recognizing distinct epitopes and (2) a different electrophoresis motility (see Fig. 2a). Interestingly, new antibodies have been developed to specifically recognize some subclasses of p53 protein isoforms. Such antibodies allow immunoprecipitation and immunofluorescence experiments (i.e., MAP4-9 recognizes the  $\Delta 133$  forms, including  $\Delta 133p53\alpha$ ,  $\Delta 133p53\beta$ , and  $\Delta 133p53\gamma$ ; KJC8 is specific for the  $\beta$  forms, including  $\Delta 133p53\beta$ ,  $\Delta 40p53\beta$ ,  $\Delta 133p53\beta$ , and  $\Delta 160p53\beta$ ) (see Figs. 1 and 2). In addition, it should be kept in mind that one could compare the expression levels of each p53 isoform only when the antibody recognizes an identical epitope per isoform. The polyclonal antibodies (SAPU or CM1), which recognize epitopes in both N- and C-terminal p53 domains, do not detect the p53 isoforms with the same affinity since some isoforms lack the N- and/or the C-terminus.

The best described biochemical activity of p53 protein isoforms is their ability to modulate p53 transcriptional activity, as described using luciferase reporter gene assays (1, 7, 9, 10). However, luciferase reporter gene assays performed to assess the impact of one protein on the transcriptional activity of a second one, requires the user to establish appropriate experimental conditions. Of note, the activity of p53 isoform on transcription can be detected and quantified even at low expression level of p53 protein isoforms, below the detection threshold by Western blotting. This suggests that the p53 isoforms are potent regulators of p53 transcriptional activity in human cells (see Fig. 3) (1, 10).

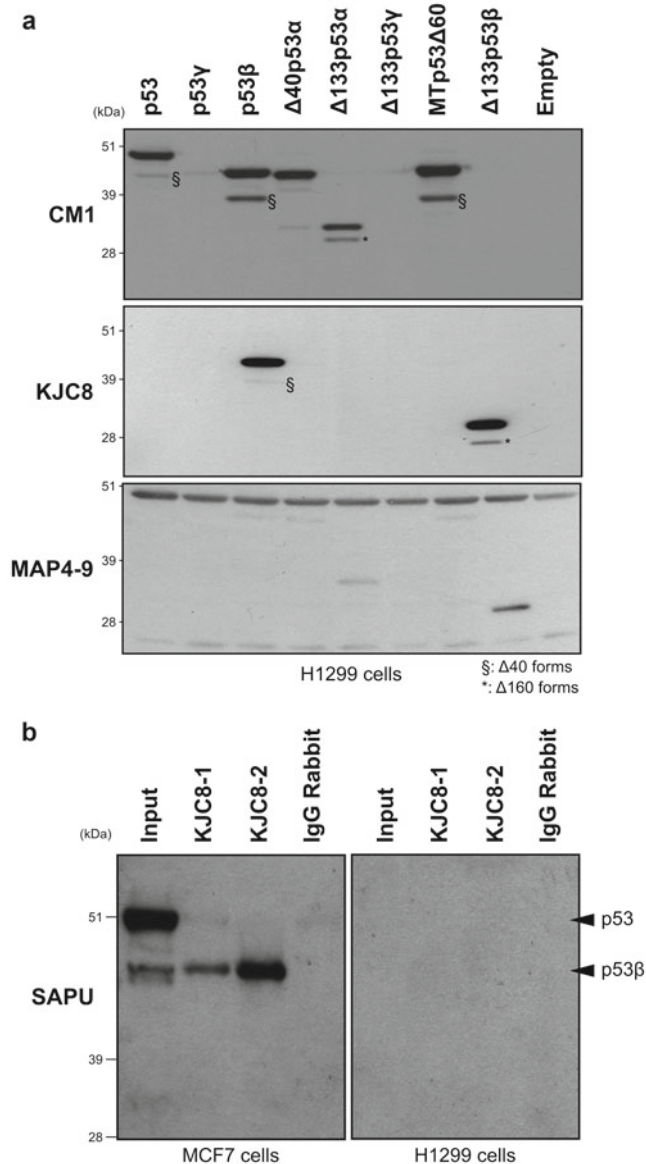
Detecting p53 isoforms at protein level remains to be improved, mainly by increasing the panel of antibodies specific to p53 isoforms. The development of these tools has been initiated with the generation of antibodies recognizing some subclasses of p53 isoforms (i.e., KJC8 or MAP4.9) and has been extended to p53 isoforms expressed in animal models, in particular, in *Drosophila melanogaster* and *Danio rerio* models (see Table 1, Fig. 4) (5).

---

## 2. Materials

### 2.1. Western Blotting

1. NP40 extraction buffer: 50 mM Tris-HCL pH 7.5; 10% glycerol; 0.1% "NP-40 Alternative" (Calbiochem); 100 mM NaCl; 0.2 mM EDTA. Store at 4°C. The buffer is supplemented extemporaneously with 1× Complete™ Protease Inhibitor Cocktail (Roche).



**Fig. 2. Detection of human p53 protein isoforms. (a)** Detection of ectopic p53 protein isoforms by Western blotting. p53-null H1299 cells were transfected with vectors expressing different p53 protein isoforms. Expression of ectopic p53 isoforms was verified by Western blotting using three different antibodies (see Subheading 2). The interpretation of a Western blot for p53 isoform expression is based on (1) distinct migration profile and (2) distinct recognition pattern given by several antibodies. For example, the band corresponding to p53 $\beta$  protein isoform (1) is located at around 47 kDa and (2) is detected by both CM1 (all isoforms) and KJC8 ( $\beta$  forms) but not by MAP4-9 ( $\Delta 133$  forms). MTp53 $\Delta 60$ : truncated mutant p53 lacking the last 60 residues; Empty: empty-expression vector; kDa: kilo Dalton; §:  $\Delta 40$  forms; \*:  $\Delta 160$  forms. **(b)** Detection of endogenous p53 protein isoforms by immunoprecipitation. Wild-type p53 MCF7 cells were used to immunoprecipitate endogenous p53 $\beta$  protein isoform using the rabbit polyclonal KJC8 antibody specific for the  $\beta$  forms, then revealed by Western blotting using the sheep polyclonal SAPU antibody recognizing all p53 protein isoforms (see Subheading 3). Immunoprecipitation of  $\beta$  forms allows a simple interpretation as compared to Western blotting. p53-null H1299 cells were used as negative control. Input: positive control (unprecipitated protein extract); IgG Rabbit: negative control (immunoprecipitation using nonspecific antibody); KJC8-1/2: 2 different batches of purified KJC8 antibodies with distinct affinity for p53 $\beta$  protein isoform.

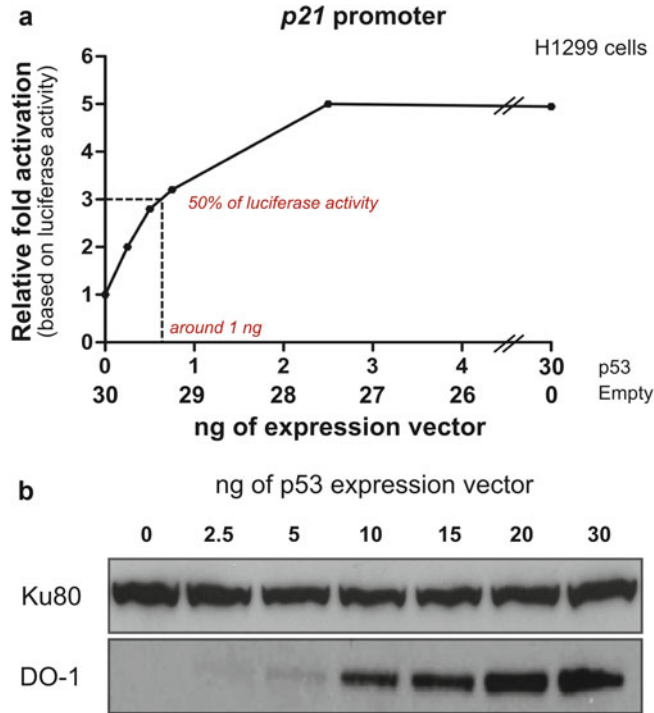


Fig. 3. Luciferase reporter gene assays to study the impact of p53 protein isoforms on p53 transcriptional activity. **(a)** Determination of the amount of p53 expression vector to transfect to obtain 50% of maximum promoter activity. The p53-null H1299 cells were co-transfected (see Subheading 4) with (1) a constant amount of Firefly luciferase reporter plasmid driven by the promoter of interest, (2) a constant amount of the control *Renilla* luciferase reporter plasmid, driven by the SV40 promoter, and (3) an increasing amount of p53 expression vector balanced with the corresponding amount of Empty-expression vector. The Firefly luciferase activity driven by the *p21* promoter is increased by a low amount of ectopic p53 protein barely detectable by western blotting **(b)** and reaches a plateau starting from 2.5 ng of transfected p53 expression vector. To be able to detect either an increase or a decrease of p53 transcriptional activity on the *p21* promoter, we transfected the amount of p53 expression vector that leads to 50% of maximum the *p21* promoter activity (here, 1 ng of pcDNA3-p53 expression vector). Empty: Empty-expression vector; Ku80: loading control.

2. 1× LDS buffer: dilution from the 4× stock solution NuPAGE® LDS Sample Buffer (Invitrogen). Store at room temperature (see Note 1).
3. DTT 1 M: 1.54 g in a final volume of 10 ml milli-Q water. Store at -20°C.
4. Electrophoresis components:
  - XCell SureLock® Mini-Cell Electrophoresis System (Invitrogen).
  - Pre-casted NuPAGE® Novex® 10% Bis-Tris Mini Gels (Invitrogen).
  - 1× Running buffer prepared from 20× NuPAGE® MOPS SDS Running Buffer (Invitrogen).
  - NuPAGE® Antioxidant (Invitrogen).

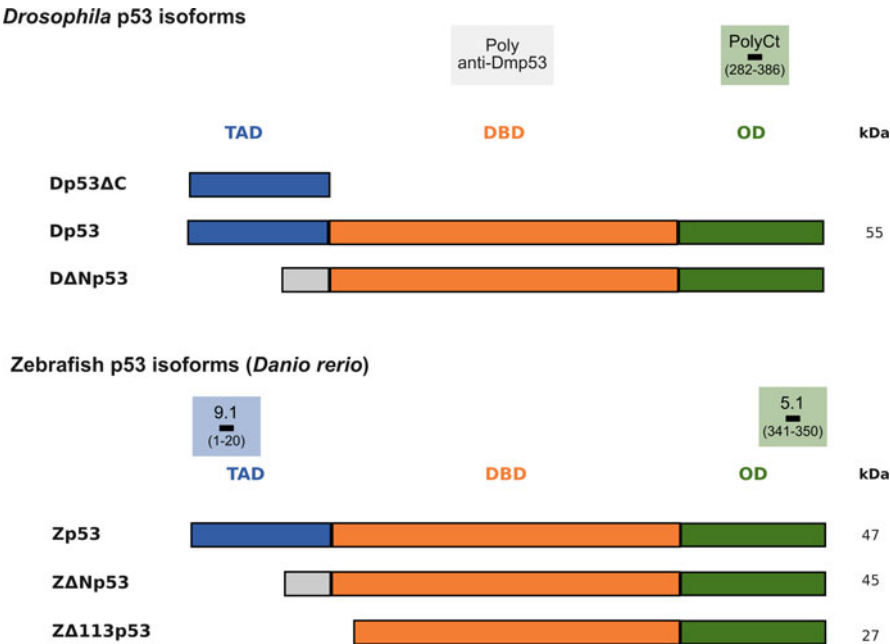


Fig. 4. p53 protein isoforms in animal models. The *TP53* gene expresses several protein isoforms conserved in different animal models. Up until now, three p53 protein isoforms have been identified in *Drosophila melanogaster* and in *Danio rerio* (zebrafish) that correspond mainly to N-truncated p53 protein isoforms. Specific antibodies have been developed to recognize *Drosophila* and zebrafish p53 isoforms. TAD: transactivation domain, blue; DBD: DNA-binding domain, orange; OD: oligomerization domain, green; gray box: residues not present in the other protein isoforms; D: *Drosophila* p53 protein; Z: zebrafish (*Danio rerio*) p53 protein; kDa: kilo Dalton, observed protein molecular weight; colored boxes: anti-p53 antibodies; Poly: polyclonal antibody; numbers within brackets: epitopes.

5. Transfer components:
- Mini Trans-Blot<sup>®</sup> Cell (Biorad).
  - 1× Transfer buffer prepared from 10× Wet blot solution (144 g glycine—30 g Tris-Base—in a final volume of 1 L milli-Q water) supplemented with 20% methanol.
  - BA83 Protran<sup>®</sup> Nitrocellulose Membrane (Whatman).
6. Immunoblotting components:
- Wash solution-1: 1× PBS prepared using 10 Dulbecco “A” tablets in a final volume of 1 L milli-Q water (Oxoid).
  - Wash solution-2: 1× PBS—0.1% Tween 20.
  - Blocking solution: 5% nonfat milk—1× PBS—0.1% Tween 20.
  - Primary antibodies are diluted in 5% milk—1× PBS—0.1% Tween 20, stored at –20°C and used up to three times (Table 1, Fig. 1).
  - Secondary Horse Radish Peroxidase (HRP)-conjugated antibodies (IgG) are purchased from Jackson Immuno

Research Laboratories, Inc., used at 1:10,000 dilution (in 5% milk—1× PBS—0.1% Tween 20). Dilutions are stored at −20°C and used only 1 time to improve detection.

7. Detection reagents:

Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare).

SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific).

Amersham Hyperfilm ECL (GE Healthcare).

## **2.2. Immuno-precipitation**

1. Materials described in Subheading 2.1.
2. RQ1 RNase-free DNase (Promega); RNase A (Qiagen).
3. *Preparation of protein G coupled to beads*: Dynabeads® Protein G (Invitrogen) according to the manufacturer's protocol ("Washing/Preparation Dynabeads").

## **2.3. Luciferase Reporter Gene Assays**

1. Transfection reagents:  
FuGENE® 6 Transfection Reagent (Roche).  
OptiMEM (Gibco™, Invitrogen).
2. Luciferase assays:  
White 96-well microplates (Greiner Bio-One).  
Dual Luciferase® Reporter Assay System (Promega) (see Note 19).

---

## **3. Methods**

### **3.1. Western Blotting**

1. Wash cells twice with cold 1× PBS, add about 100 µl of NP40 extraction buffer directly into dishes, keep on ice, and scrape off the cells with a cell scraper (see Notes 1 and 2).
2. Collect cell lysate into a tube, keep on ice (see Note 3).
3. Pass cell lysate at least five times through a syringe with a 23 G needle to fragment cell membranes (see Note 3).
4. Spin cell lysate for 10 min, 13,800×g, at 4°C.
5. Conserve the supernatant in a fresh tube (see Note 3).
6. Determine protein concentration (see Note 4).
7. Prepare 10–30 µg of protein sample supplemented with 0.1 M DTT and 1× LDS buffer (Invitrogen) (see Note 5).
8. Heat protein samples at 95°C for 5 min.
9. Load between 10 and 30 µg of protein sample per well on pre-casted NuPAGE 10% Bis-Tris polyacrylamide gels (Invitrogen) and separate proteins using 1× MOPS Running Buffer



supplemented with 0.2% Antioxidant as described by the manufacturer (Invitrogen) at 80 V for 2–3 h (see Note 6).

10. Transfer proteins overnight (25 mA, 11 V, about 14 h) onto the nitrocellulose membrane using 1× Transfer buffer supplemented with 20% methanol (see Note 7).
11. Block membrane with 5% nonfat milk—1× PBS—0.1% Tween 20 for 20 min by gentle shaking (see Note 8).
12. Place the membrane on the bench protected with a piece of saran film and add directly the primary antibody onto the membrane (1 ml per 50 cm<sup>2</sup>) (see Note 9).
13. Cover to avoid evaporation and incubate for 1 h (see Note 10).
14. Wash with 5% nonfat milk—1× PBS—0.1% Tween 20 for 10 min by gentle shaking.
15. Repeat steps 12 and 13 for the secondary antibody (see Table 1).
16. Wash the membrane twice with 1× PBS—0.1% Tween 20 for 7 min, followed by an additional 7 min wash with 1× PBS.
17. Incubate membrane with Amersham ECL Western blotting detection system (GE Healthcare) or with SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific), according to the manufacturer's recommendations, by directly adding the reagent onto the membrane placed on the bench (see Note 11).
18. Exposure time of Hyperfilm to membrane varies according to the p53 protein isoform of interest (see Note 12).
19. Development (for example, using the Konica Medical Film Processor).
20. Analysis of p53 protein isoform expression (see Note 13) (see Fig. 2a).

### **3.2. Immuno-precipitation**

1. Seed cells at least 24 h prior to immunoprecipitation to obtain up to 80% confluent cells the day of harvest (e.g.,  $2 \times 10^7$  of MCF-7 cells in a 125 ml flask).
2. Wash cells twice with cold 1× PBS.
3. Add 3 ml of NP40 extraction buffer extemporaneously supplemented with 1× Complete Mini protease inhibitor and 1 mM DTT directly onto cells, keep on ice.
4. Scrape off cells from flask and leave flask to rock gently for 15 min at 4°C.
5. Transfer cell lysate to 5 ml tubes, keep in ice (see Note 3).
6. Syringe the cell lysate at least ten times through a 23 G needle (see Note 3).
7. Transfer into several 1.5 ml tubes, keep in ice, and spin at  $11,800 \times g$  for 10 min at 4°C.

8. Aliquot 500  $\mu$ l of supernatant per tube and treat with 10 U of DNase RQ (Promega) and 35 U of RNase A (Qiagen) for 20 min at room temperature on a rotating wheel (see Note 3).
9. Pre-clear the lysate by adding 50  $\mu$ l of prepared protein G coupled to beads for 15 min at 4°C on a rotating wheel.
10. Using the magnet, transfer the lysate into a new tube (see Note 3).
11. Determine protein concentration (see Note 4).
12. Use 5 mg of total protein lysate concentrated in 1 ml per immunoprecipitation (see Notes 14 and 15).
13. Add the required amount (suggested up to 10  $\mu$ g) of primary antibody to the total protein lysate and incubate for 2 h at 4°C on a rotating wheel (see Note 16) (see Table 1).
14. Add 100  $\mu$ l of prepared protein G coupled to beads for 2 h at 4°C on a rotating wheel.
15. Using the magnet, discard the supernatant.
16. Wash beads four times with 500  $\mu$ l of NP40 extraction buffer extemporaneously supplemented with 1 $\times$  Complete Mini protease inhibitor using the magnet (see Note 17).
17. Wash beads twice with 500  $\mu$ l 1 $\times$  PBS using the magnet.
18. Suspend the beads in 20  $\mu$ l of 1 $\times$  LDS Buffer (Invitrogen) and heat for 10 min at 70°C.
19. Using the magnet, transfer the supernatant in a fresh tube (see Note 5).
20. Perform Western blot as described in Subheading 2.2 starting from step 9 using the totality of protein samples.
21. Analysis of immunoprecipitation (see Note 18) (see Fig. 2b).

### **3.3. Luciferase Reporter Gene Assays**

1. Seed  $3 \times 10^4$  cells per well in 24-well plates 24 h prior to transfection in a final volume of 500  $\mu$ l.
2. Prepare a mix of plasmids containing the amount of plasmids determined during the pre-experimental process of titration (see Fig. 3). For example, using the *p21*-luciferase reporter gene in p53-null H1299 cells, mix 2 ng/well of *Renilla* luciferase reporter plasmid, 200 ng/well of Firefly luciferase reporter plasmid, and 1 ng/well of expression vector (see Note 20).
3. Transfect plasmid mix using FuGENE® 6 Transfection Reagent (Roche) with OptiMEM® (Invitrogen) according to the manufacturer's protocol (ratio FuGENE:DNA = 3:1) using a final volume of 100  $\mu$ L. Prior to transfection, replace media with 400  $\mu$ L of fresh complete media.
4. Incubate cells under normal conditions for 24 h.
5. Wash cells twice with cold 1 $\times$  PBS and measure luciferase activity as described by the manufacturer using 10  $\mu$ L of the whole

cell lysate and 30  $\mu$ L of both luciferase substrates (5 s time delay and 10 s measurement, for example, using the Luminometer (EG&GBERTHOLD, Microplate Luminometer LB 96 V)).

6. Perform analysis (see Note 21).

---

## 4. Notes

1. Cells can be directly lysed in 1 $\times$  LDS buffer (Invitrogen) instead of using the NP40 extraction buffer (no need for items 4 and 5 of Subheading 2.2). However, detection of p53 protein isoforms can be less efficient using this alternative extraction method because of post-translational modifications on some epitopes.
2. Use one volume of NP40 extraction buffer corresponding to cell confluence (i.e., add 80  $\mu$ L of buffer onto 80% confluent cells).
3. Experiment can be stopped at this stage and samples can be stored at  $-80^{\circ}\text{C}$ .
4. Different protocols can be used. We recommend the Qubit<sup>®</sup> 2.0 Fluorometer (Invitrogen) using the Qubit Protein Assay (Invitrogen), which provides a sensible and accurate detection.
5. Experiment can be stopped at this stage and samples can be stored at  $-20^{\circ}\text{C}$ .
6. Detection of p53 protein isoforms requires sufficient migration to dissociate proteins with closed molecular weights (see Fig. 1). We recommend to stop the running process when the migration front has just dissolved in the lower buffer chamber (keep the 15 kDa band of the protein molecular weight marker in the gel).
7. Alternatively, transfer can be performed for 1 h 30 min at 200 mA at  $4^{\circ}\text{C}$ .
8. The blocking process may vary from 20 min to 1 h without any alteration of the detection.
9. Addition of antibodies directly onto the membrane, rather than using the classical “bath” process, limits antibody consumption and improves the immunoreaction. Usually, use 1  $\mu$ g of antibody per ml diluted in 5% milk—1 $\times$  PBS—0.1% Tween 20.
10. Alternatively, membranes can be incubated within a bath of 5 ml primary antibody overnight at  $4^{\circ}\text{C}$  under gentle shaking.
11. In most cell lines, Amersham ECL Western blotting detection system (GE Healthcare) is sufficient to detect N-terminal TA

and  $\Delta 40$  forms (see Fig. 2a, CM1 antibody), while SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific) is required to detect  $\Delta 133$  and  $\Delta 160$  forms (see Fig. 2a, MAP4-9 antibody).

12. Time of exposure using Amersham Hyperfilm varies according to cell lines and to the p53 protein isoform of interest and has to be determined by the user. Exposure time may vary from 1 min to 1 h.
13. Regarding the analysis of p53 protein isoforms, the interpretation of Western blotting is based on (a) the molecular weight and (b) the detection pattern of distinct antibodies (see Fig. 2a, e.g., p53 $\beta$  at about 47 kDa and detected with CM1 (specific for all isoforms) and KJC8 (specific for  $\beta$  forms) but not with MAP4-9 (specific for  $\Delta 133$  forms)). However, comparison of expression levels between p53 isoforms requires the use of an antibody, which recognizes an identical epitope, at a given time exposure using the same detection reagent (Fig. 2a, e.g., comparison of p53 $\beta$  and  $\Delta 133$ p53 $\beta$  expression levels using KJC8 ( $\beta$  forms) but not CM1 (all isoforms)). To assess the specificity of the antibody, it is necessary to first compare the detection of protein issued from cells transfected with vector expressing ectopic p53 and with an Empty-expression vector. Of note, internal initiations of translation have been described for TAp53 forms and  $\Delta 133$  forms leading to  $\Delta 40$  and  $\Delta 160$  forms, respectively (7, 8). Thus, ectopic expression of TA and  $\Delta 133$  forms results in the detection of a doublet (see Fig. 2a). The clear identification of a p53 protein isoform by Western blotting requires at least one of the following controls: (a) use of a panel of distinct antibodies; (b) loading of proteins extracted from cells transfected with p53 isoform expression vector; and (c) use of siRNA specifically targeting p53 isoforms.
14. To immunoprecipitate p53 isoforms, 1–5 mg of total protein lysate is required and is prepared in a final volume of 500–1,000  $\mu$ l.
15. For each sample, use the same amount of protein extract to perform an immunoprecipitation using your antibody of interest and an immunoprecipitation using a control antibody (i.e., IgG mouse if the primary antibody of interest is a mouse antibody, IgG rabbit if using a primary rabbit antibody, etc). About 50  $\mu$ g of protein extract is stored at  $-80^{\circ}\text{C}$  and used as the input control (see Fig. 2b).
16. The amount of primary antibody varies according to the expression level of the p53 isoform of interest, the number of epitopes per isoform for one considered antibody, and the specificity of the antibody (see Table 1, Fig. 1).
17. Do not add DTT at this step.

18. If the right controls are performed, interpretation of immunoprecipitation is easier than the one of Western blotting since analysis is based only on the distinct molecular weight of p53 protein isoforms. Immunoprecipitation using two different anti-p53 antibodies is an ideal experiment to identify p53 isoforms. However, immunoprecipitation cannot be used to compare protein expression levels under different experimental conditions.
19. As compared to other reporter gene assays (i.e., Chloramphenicol Acetyl Transferase assays), the luciferase reporter gene assay is highly recommended to study promoter activity since the luciferase enzymes have a short half-life, as well as a rapid enzymatic activity, and current luminometer kits allow the detection of small amounts of luciferase enzyme (from  $10^{-20}$  molecule).
20. The amount of plasmids (Basic luciferase, Firefly luciferase, *Renilla* luciferase, and expression vector) should be carefully predetermined prior to experimentation, because of the specificity of each cell line, each promoter, and each transcription factor. First, the amount of the promoterless Basic luciferase reporter plasmid, used to normalized the experiments (see Note 21), should not yield ten times more luciferase activity than the background light given by non-transfected cells. Second, the optimal amount of Firefly luciferase reporter plasmid driven by the promoter of interest should yield two to three times more luciferase activity than the promoterless Basic luciferase reporter plasmid. Third, the amount of *Renilla* luciferase reporter plasmid, which is driven by the constitutive promoter SV40, corresponds to 1/100 of the amount of Firefly luciferase reporter plasmid. We do not recommend the use of the thymidine kinase promoter as in internal control since it contains some p53 responsive elements. Finally, the amount of p53 expression vector should be carefully determined. To study the regulation of p53 transcriptional activity on a promoter of interest, the experiment should be performed at 50% of the maximum promoter activity allowing thus the detection of both decreased and increased p53 transcriptional activity. Usually, a promoter activity in response to increasing amount of p53 has two phases (see Fig. 3): a “linear” phase, where the promoter activity is proportional to the amount of p53; and a “plateau” phase, corresponding to the maximal activity of a given promoter in response to p53. A titration experiment allows the determination of the amount of p53 expression vector to transfect to obtain 50% of maximum promoter activity. Importantly, it is crucial to balance the amount of p53 expression vector with the corresponding Empty-expression vector in order to transfect a constant amount of

DNA expression vector (e.g., pcDNA3-p53 + pcDNA3-Empty = 30 ng) (see Fig. 3).

21. Results of luciferase assays are usually presented as an average of at least three independent experiments performed in triplicate. In a first step, each independent experiment should be carefully normalized. This “intra-”normalization using the *Renilla* luciferase activity avoids misinterpretation due to variation in pipetting, transfection efficiency, or cell death (Firefly/*Renilla*). In addition, each condition is expressed as a ratio compared to the promoterless Basic luciferase reporter plasmid ( $[\text{Firefly}/\text{Renilla}]_{\text{condition1}}/[\text{Firefly}/\text{Renilla}]_{\text{Basic}}$ ). As for the “intra-”normalization, this “inter-”normalization avoids misinterpretation due to variation in cell passage, transfection efficiency, etc. In a second step, average can be calculated from the normalized values. Important controls to consider are (1) “Basic” condition (co-transfection of promoterless Basic luciferase reporter plasmid and Empty-expression vector) to allow the “inter-”normalization; (2) “Empty” condition (co-transfection of the Firefly luciferase reporter plasmid and Empty-expression vector) to allow determination of the intrinsic promoter activity and to be used as a negative control when an ectopic protein is co-expressed; (3) “Plateau” condition (co-transfection of the Firefly luciferase reporter plasmid with the amount of expression vector reaching the plateau of fold-activation, see Note 20) to be used as a positive control when an ectopic protein is co-expressed. Of note, when transfecting increasing amount of expression vector, the total amount of DNA corresponding to the expression vector should be kept constant by balancing with Empty-expression vector (e.g., pcDNA3-p53 + pcDNA3-Empty = 30 ng) (see Fig. 3).

---

## Acknowledgments

This work was supported by Cancer Research UK (C8/A6613). V.M. is supported by Breast Cancer Campaign, M.P.K., K.F., A.D., and J.C.B. are supported by Cancer Research UK.

## References

1. Bourdon J-C, Fernandes K, Murray-Zmijewski F, Liu G, Diot A, Xirodimas DP, Saville MK, Lane DP (2005) p53 isoforms can regulate p53 transcriptional activity. *Genes Dev* 19:2122–2137
2. Aoubala M, Murray-Zmijewski F, Khoury MP, Fernandes K, Perrier S, Bernard H, Prats AC, Lane DP, Bourdon J-C (2011) p53 directly transactivates  $\Delta 133\text{p}53\alpha$ , regulating cell fate outcome in response to DNA damage. *Cell Death Differ* 18:248–258
3. Fujita K, Mondal AM, Horikawa I, Nguyen GH, Kumamoto K, Sohn JJ, Bowman ED, Mathe EA, Schetter AJ, Pine SR, Ji H, Vojtesek

- B, Bourdon J-C, Lane DP, Harris CC (2009) p53 isoforms Delta133p53 and p53beta are endogenous regulators of replicative cellular senescence. *Nat Cell Biol* 11:1135–1142
4. Marcel V, Vijayakumar V, Fernandez-Cuesta L, Hafsi H, Sagne C, Hautefeuille A, Olivier M, Hainaut P (2010) p53 regulates the transcription of its Delta133p53 isoform through specific response elements contained within the *TP53* P2 internal promoter. *Oncogene* 29:2691–2700
  5. Marcel V, Dichtel-Danjoy ML, Sagne C, Hafsi H, Ma D, Ortiz-Cuaran S, Olivier M, Hall J, Mollereau B, Hainaut P, Bourdon J-C (2011) Biological functions of p53 isoforms through evolution: lessons from animal and cellular models. *Cell Death Differ* 18(12):1815–24
  6. Khoury MP, Bourdon J-C (2010) The isoforms of the p53 protein. *Cold Spring Harb Perspect Biol* 2:a000927
  7. Courtois S, Verhaegh G, North S, Luciani MG, Lassus P, Hibner U, Oren M, Hainaut P (2002) DeltaN-p53, a natural isoform of p53 lacking the first transactivation domain, counteracts growth suppression by wild-type p53. *Oncogene* 21:6722–6728
  8. Marcel V, Perrier S, Aoubala M, Ageorges S, Groves MJ, Diot A, Fernandes K, Tauro S, Bourdon J-C (2010)  $\Delta$ 160p53 is a novel N-terminal p53 isoform encoded by  $\Delta$ 133p53 transcript. *FEBS Lett* 584:4463–4468
  9. Yin Y, Stephen CW, Luciani MG, Fähræus R (2002) p53 stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. *Nat Cell Biol* 4:462–467
  10. Murray-Zmijewski F, Lane DP, Bourdon J-C (2006) p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death Differ* 13:962–972
  11. Ollman M, Young JM, Di Como CJ, Karim F, Belvin M, Robertson S, Whittaker K, Demsky M, Fisher WW, Buchman A, Duyk G, Friedman L, Prives C, Kopczynski C (2000) *Drosophila* p53 is a structural and functional homolog of the tumor suppressor p53. *Cell* 101:91–101
  12. Brodsky MH, Nordstrom W, Tsang G, Kwan E, Rubin GM, Abrams JM (2000) *Drosophila* p53 binds a damage response element at the *reaper* locus. *Cell* 101:103–113
  13. Lee K-C, Goh WLP, Xu M, Kua N, Lunny D, Wong JS, Coomber D, Vojtesek B, Lane EB, Lane DP (2008) Detection of the p53 response in zebrafish embryos using new monoclonal antibodies. *Oncogene* 27:629–640



<http://www.springer.com/978-1-62703-235-3>

p53 Protocols

Deb, S.; Deb, S.P. (Eds.)

2013, X, 238 p., Hardcover

ISBN: 978-1-62703-235-3

A product of Humana Press