DCode™ Control Reagent Kit for DGGE, CDGE, TTGE

Instruction Manual

Catalog Number
170-9150

For Technical Service
Call Your Local Bio-Rad Office or
in the U.S. Call **1-800-4BIORAD**
(1-800-424-6723)

BIO-RAD

Table of Contents

* The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffmann-La Roche.

Section 1
Introduction

The DCode control kit for DGGE (Denaturing Gradient Gel Electrophoresis), CDGE (Constant Denaturing Gel Electrophoresis), and TTGE (Temporal Temperature Gradient Electrophoresis) provides reagents that are used to prepare mutant and wild-type DNA for denaturing gel electrophoresis with the DCode Universal Mutation Detection System.[1, 2, 7] In a denaturing gradient acrylamide gel, double stranded DNA is subjected to an increasing denaturant environment and will melt in discrete segments, called melting domains. The melting temperature (T_m) of these domains is sequence specific. When the T_m of the lowest melting domain is reached, the DNA will become partially melted, creating branched molecules. Partial melting of the DNA reduces its mobility in a polyacrylamide gel. Since the T_m of a particular melting domain is sequence specific, the presence of a mutation will alter the melting profile of that DNA when compared to wild-type. DNA containing mutations will encounter the mobility shifts caused by partial melting at a different position in the gel than the wild-type.

In DGGE, the denaturing environment is created by a combination of uniform temperatures typically between 50 and 65 °C and a linear denaturant gradient formed with urea and formamide. The denaturing gradient may be formed perpendicular or parallel to the direction of elec-

trophoresis. After a mutation has been identified, CDGE gels can be used to rapidly screen samples for the presence of a mutation. In CDGE, the denaturant concentration found to give optimal resolution from the gradient gel is held constant. TTGE exploits the principles upon which DGGE is based, without the need for a chemical denaturing gradient. Only urea in the gel and a temporal temperature gradient of the buffer are used as denaturants. The DNA samples are electrophoresed in a gel where the temperature is increased linearly through time. The result is a linear temperature gradient over the time course of the electrophoresis run.

Since analysis of DNA samples on a perpendicular and parallel denaturing gradient gel is highly dependent on a good gradient, this kit will allow you to check for proper casting of a perpendicular and parallel denaturant gradient gel before using your own samples. Using the Model 475 Gradient Delivery System will help you form reproducible and linear gradients. The controls can also be used to test the TTGE method with the DCode system.

The DCode control kit for DGGE, CDGE, and TTGE contains human genomic wild-type DNA and mutant DNA. The mutant DNA is identical to the wild-type DNA, except for a single G to A mutation (PCR fragment base number 138) in exon 8 of the p53 gene.[6] The PCR reaction will produce a 191 bp fragment. Also included in the kit are two oligonucleotide primers, a 20 base pair primer and a 60 base pair primer. The 60 base pair primer contains a

40 base pair GC clamp. Attachment of a GC clamp creates a high melting domain within the DNA fragment and allows the detection of most mutations.[3,4,5] The control kit also contains a 2x gel loading dye. There are sufficient amounts of DNA and primers for five 100 µl PCR reactions for both the mutant and wild-type DNA.

1.1 Kit Components

Item	Concentration	Amount	Volume
Mutant DNA	100 ng/µl	500 ng	5 µl
Wild-type DNA	100 ng/µl	500 ng	5 µl
Primer A	25 pmol/µl	500 pmol	20 µl
Primer B	25 pmol/µl	250 pmol	10 µl
Gel Loading Dye	2x	————	1 ml

Sequence of Primer A: 5'- ATC CTG AGT AGT GGT AAT CT -3'

Sequence of Primer B: 5'- GCG GGC GGC GCG GGG CGC GGG CAG GGC GGC GGG GGC GGG CTA CCT CGC TTA GTG CTC CCT -3'

2x Gel Loading Dye: 2 mM EDTA pH 8.0, 70% glycerol, 0.05% xylene cyanol, and 0.05% bromophenol blue

1.2 Additional Supplies Required

Taq DNA polymerase enzyme
10x *Taq* polymerase buffer
10 mM dNTPs
Sterile water
Thin-walled microfuge tubes–200 µl or 500 µl size
Sterile aerosol tips

1.3 Storage Conditions

All kit components should be stored at -20 °C, except the 2x gel loading dye which can be stored at room temperature. The shelf life of the kit stored at -20 °C is 1 year.

Section 2
PCR Reaction

Note: It is extremely important that solutions and materials used during PCR set-up are not exposed to amplified DNA to avoid contamination during amplification.

1. Add the components listed below to 200 µl or 500 µl thin-walled microfuge tubes and mix.

	Tube 1 Wild-type DNA	**Tube 2 Mutant DNA**
Control DNA	1.0 µl	1.0 µl
Primer A	2.0 µl	2.0 µl
Primer B	1.0 µl	1.0 µl
10x *Taq* polymerase buffer	10.0 µl	10.0 µl
10 mM dNTPs	2.0 µl	2.0 µl
Taq DNA polymerase enzyme (5 U/µl)	0.5 µl	0.5 µl
Sterile water	83.5 µl	83.5 µl
Total volume	100.0 µl	100.0 µl

Note: 10x *Taq* polymerase buffer contains 100 mM Tris-HCl, 500 mM KCl, and 25 mM $MgCl_2$ (pH 9.2). If desired, add a third tube for a negative control.

2. Place the tubes into a thermocycler and enter the following parameters:

Step 1	94 °C	4'	x 1 cycle
Step 2	94 °C	45"	x 35 cycles
	55 °C	45"	
	72 °C	45"	
Step 3	72 °C	10'	x 1 cycle

Note: For thick-walled tubes, adjust all step 2 cycle times from 45 seconds to 1 minute.

3. To check the product, add 5 µl of the amplified DNA to 1 µl of the 2x gel loading dye and run on a 4% AmpliSize agarose gel (catalog number 162-0144). Run a DNA size standard, such as Bio-Rad's 20 bp Molecular Ruler catalog number 170-8201), on the gel to approximate the size of the product. The size of the PCR product should be 191 base pairs.
4. Store the amplified DNA at 4 °C. For long term storage (> 2 weeks), store the amplified DNA at -20 °C.

Section 3
Perpendicular Denaturing Gradient Gel

Perpendicular denaturing gradient gel uses a range of denaturants to separate the mutant and wild-type DNA fragments. Both the mutant and wild-type DNA are electrophoresed on a perpendicular denaturing gradient gel,

and the DNA pattern will form an "S" shape. There should be a split (difference in migration) between the mutant and wild-type DNA (Figure 1).

Note: Refer to the DCode Universal Mutation Detection System manual for information on casting and running perpendicular denaturing gradient gels on the DCode Universal Mutation Detection System.

1. To a microfuge tube, add 50 µl (~1.5–3 µg) of the amplified mutant DNA, 50 µl of the amplified wild-type DNA, and 100 µl of the 2x gel loading buffer and mix.
2. Cast a 10% acrylamide/bis gel (37.5:1) in 1x TAE buffer with a perpendicular denaturant gradient of 20–70%.
3. The run conditions for the perpendicular denaturing gradient gel are as follows:

Buffer	1x TAE
Buffer temperature	56 °C
Voltage	130 V
Run time	2.0 hours

Note: Preheat the running buffer prior to a run.

4. Load 200 µl of the sample into the prep well of the perpendicular denaturing gradient gel and run the gel under the conditions given above.
5. After the run is completed, stain the gel in a 50 µg/ml ethidium bromide in 1x TAE buffer solution for about 3 minutes. Destain the gel in 1x TAE buffer for about 20 minutes. Visualize and photograph the gel on a UV

transilluminator (Gel Doc™ system 1000, catalog numbers 170-7520 through 170-7527 or Bio-Rad Polaroid Gel Documentation System, catalog numbers 170-3742 through 170-3749).

Note: Use of the running buffer to handle the gel after a run, reduces gel swelling.

6. The gel results should look similar to that in Figure 1. A separation should be formed between the mutant and wild-type DNA. This separation is caused by the mutant DNA melting sooner than the wild-type DNA.

Fig. 1. Separation of mutant and wild-type DNA electrophoresed on a 20–70% perpendicular denaturing gradient gel. The gel was 10% acrylamide:bis (37.5:1), run at 130 volts in 1x TAE buffer, heated at 56 °C, for 2 hours.

Section 4
Parallel Denaturing Gel

For a parallel denaturing gel, the denaturant is parallel to the electrophoresis direction. A time course run is initially done for parallel gels to find the optimal run time for the mutant and wild-type DNA samples. At the optimal denaturant on the gel with the samples in this kit, the mutant DNA will migrate slower than the wild-type DNA, allowing the DNA fragments to be resolved

Note: Refer to the DCode Universal Mutation Detection System manual for information on casting and running parallel denaturing gels on the DCode Universal Mutation Detection System.

1. To three microfuge tubes, add the amplified mutant and wild-type DNA as follows:

Tube #	Sample	Volume	Concentration	2x Gel Loading Dye
1	mutant	5 µl	~180–300 ng	5 µl
2	wild-type	5 µl	~180–300 ng	5 µl
3	mutant + wild-type	5 µl each	~180–300 ng each	10 µl

2. Cast a 8% acrylamide/bis gel (37.5:1) in 1x TAE buffer with a parallel denaturant gradient of 40–65%.

3. The run conditions for the parallel denaturing gradient gel are as follows:

Buffer	1x TAE
Buffer temperature	60 °C
Voltage	150 V
Run time	2.5 hours

Note: Preheat the running buffer prior to a run.

4. Load 10 µl of the mutant sample into lane 1. Load 10 µl of the wild-type sample into lane 2. In the third lane, load 20 µl of the tube containing amplified mutant and wild-type samples. Run the gel under the conditions given in step 3.
5. After the run is completed, stain the gel in a 50 µg/ml ethidium bromide in 1x TAE buffer solution for about 3 minutes. Destain the gel in 1x TAE buffer for about 20 minutes. Visualize and photograph the gel on a UV transilluminator (Gel Doc system 1000 catalog numbers 170-7520 through 170-7527 or Bio-Rad Polaroid Gel Documentation System, catalog numbers 170-3742 through 170-3749).

Note: Use of the running buffer to handle the gel after a run reduces gel swelling.

6. The gel result should look similar to that in Figure 2. The mutant sample will migrate slower than the wild-type sample. In this case, the mutant DNA melts sooner than the wild-type DNA.

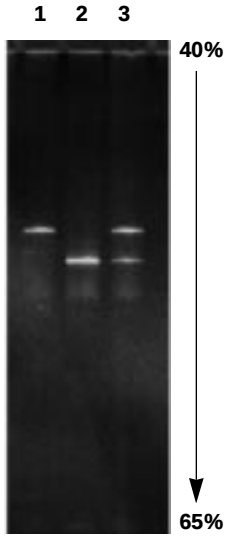

Fig. 2. Separation of mutant and wild-type DNA electrophoresed on a 40–65% parallel denaturing gradient gel. The gel was 8% acrylamide:bis (37.5:1), run at 150 volts in 1x TAE buffer, heated at 60 °C, for 2.5 hours. Lane 1, mutant DNA, lane 2, wild-type DNA, and lane 3, mutant and wild-type DNA.

Section 5
Constant Denaturing Gel

The mutant and wild-type amplified DNA can also be run on a constant denaturing gel (CDGE). For a constant denaturing gel, a single denaturant concentration is used to melt the fragments. The concentration of denaturant to use for a CDGE is determined at the maximum split between wild-type and mutant DNA, as seen in a perpendicular or parallel denaturing gradient gel.

Note: Refer to the DCode Universal Mutation Detection System manual for information on casting and running a constant denaturing gel on the DCode Universal Mutation Detection System.

1. To three microfuge tubes, add the amplified mutant and wild-type DNA as follows:

Tube #	Sample	Volume	Concentration	2x Gel Loading Dye
1	mutant	5 µl	~180–300 ng	5 µl
2	wild-type	5 µl	~180–300 ng	5 µl
3	mutant + wild-type	5 µl each	~180–300 ng each	10 µl

2. Cast a 10% acrylamide/bis gel (37.5:1) in 1x TAE buffer with a constant denaturant of 51%.

3. The run conditions for the constant denaturing gradient gel are as follows:

Buffer	1x TAE
Buffer Temperature	56 °C
Voltage	130 V
Run Time	2.5 hours

Note: Preheat the running buffer prior to a run.

4. Load 10 µl of the mutant sample into lane 1. Load 10 µl of the wild-type sample into lane 2. In the third lane, load 20 µl of the tube containing amplified mutant and wild-type samples. Run the gel under the conditions given in step 3.
5. After the run is completed, stain the gel in a 50 µg/ml ethidium bromide in 1x TAE buffer solution for about 3 minutes. Destain the gel in 1x TAE buffer for about 20 minutes. Visualize and photograph the gel on a UV transilluminator (Gel Doc system 1000, catalog numbers 170-7520 through 170-7527 or Bio-Rad Polaroid Gel Documentation System, catalog numbers 170-3742 through 170-3749).

Note: Use of the running buffer to handle the gel after a run reduces gel swelling.

6. The gel result should look similar to that in Figure 3. The mutant sample will migrate slower than the wild-type sample, because the mutant DNA melts sooner than the wild-type.

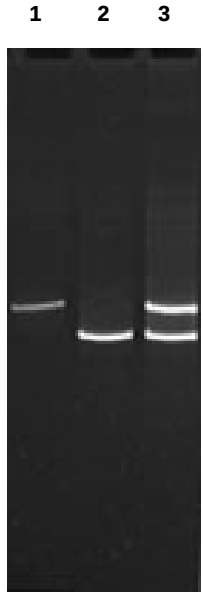

Fig. 3. Separation of mutant and wild-type DNA electrophoresed on a 51% constant denaturing gel. The gel was 8% acrylamide:bis (37.5:1), run at 130 volts in 1x TAE buffer, heated at 56 °C, for 2.5 hours. Lane 1, mutant DNA, lane 2, wild-type DNA, and lane 3, mutant and wild-type DNA.

Section 6
Temporal Temperature Gradient Gel

The mutant and wild-type amplified DNA can also be run on a temporal temperature gradient gel (TTGE). DNA is electrophoresed in a gel where the temperature is linearly increased through time. Electrophoretic mobility is decreased when the DNA molecules reach the optimal denaturation temperature in the urea gel. Using the samples in this kit, the mutant DNA will migrate slower than the wild-type DNA, thus resolving the two samples.

Note: Refer to the DCode Universal Mutation Detection System manual for information on casting and running temporal temperature gradient gels.

1. To three microfuge tubes, add the amplified mutant and wild-type DNA as follows:

Tube #	Sample	Volume	Concentration	2x Gel Loading Dye
1	mutant	5 µl	~180–300 ng	5 µl
2	wild-type	5 µl	~180–300 ng	5 µl
3	mutant + wild-type	5 µl each	~180–300 ng each	10 µl

2. Cast a 8% acrylamide/bis gel (37.5:1) containing 7 M urea and 1.5x TAE buffer.

3. The run conditions for the TTGE gel are as follows:

Buffer	1.5x TAE
Temperature range	63–66 °C
Ramp rate	1 °C/hr
Voltage	130 V
Run time	3 hours

Note: Preheat the running buffer prior to a run.

4. Load 10 µl of the mutant sample into lane 1. Load 10 µl of the wild-type sample into lane 2. In the third lane, load 20 µl of the tube containing amplified mutant and wild-type samples. Run the gel under the conditions mentioned in step 3.
5. After the run is completed, stain the gel in a 50 µg/ml ethidium bromide in 1.5x TAE buffer solution for about 3 minutes. Destain the gel in 1.5x TAE buffer for about 20 minutes. Visualize and photograph the gel on a UV transilluminator (Gel Documentation System 1000 catalog number 170-7520 through 170-7527 or Bio-Rad Polaroid Gel Documentation System, catalog numbers 170-3742 through 170-3749).

Note: Use of the running buffer to handle the gel after a run, reduces gel swelling.

6. The gel results should look similar to that in Figure 4. The mutant sample will migrate slower than the wild-type sample, because the mutant DNA melts sooner than the wild-type DNA.

Fig. 4. Separation of mutant and wild-type DNA electrophoresed on a TTGE gel. The gel was 8% acrylamide:bis (37.5:1) containing 7 M urea, run at 130 volts for 3 hours in 1.5x TAE buffer, temperature range from 63–66 °C, and ramp rate of 1 °C/hr. Lane 1, mutant DNA, lane 2, wild-type DNA, and lane 3, mutant and wild-type DNA. Excess primers may be seen near the bottom of the gel.

Section 7
Troubleshooting

Refer to the DCode Universal Mutation Detection System and Model 475 Gradient Delivery System for more troubleshooting details.

7.1 PCR

Problem	Cause	Solution
Faint, visible bands, 20 or 60 bp in size	Unused primers	1. Not a problem.
No 191 bp band	No template DNA	1. Make sure template DNA is added to PCR reaction.
	Inactive/missing enzyme	2. Make sure active enzyme is added.
	Missing dNTPs	3. Make sure all four dNTPs are used in PCR reaction.
	Missing primer/primers	4. Make sure both primers are used in PCR reaction.
Numerous bands	Nonspecific priming	1. Perform "hot start" PCR.[8]

7.2 Perpendicular Denaturant Gradient Gel

Problem	Solution
Only a single band is seen in the "S" curve when at least two bands are expected	1. Mix normal and mutant DNA prior to the run.
	2. Check PCR reaction products for mutant and normal DNA.
Unknown faint bands	1. Do "hot start" PCR.[8]
Poor gradient. "S" curve not fully seen	1. Make sure high and low density denaturing gel solutions are used on the correct side of the gradient former.
	2. Check concentrations of high and low density denaturing gel solutions.
	3. Check that correct buffer temperature was used.
	4. Insure after casting gel that the tilt rod was at level position.

7.3 Parallel Denaturant Gradient Gel

Problem	Solution
Fuzzy DNA bands	1. Clean wells prior to loading samples. 2. Use matching comb and spacer thickness. 3. Allow gel to polymerize for 60 minutes.
Smear at top of gel	1. Probably genomic DNA; this is OK.
Bands don't migrate far into gel	1. Check buffer concentration. Buffer concentration may be high. 2. Check acrylamide concentration. Acrylamide concentration may be too high. 3. Check gel solution concentrations. Make new gel solutions. Denaturant concentration may be too high. 4. Check voltage to DCode Universal Mutation Detection System. Voltage may be too low.
Streaking or DNA spikes in gel	1. Impurities in acrylamide. Filter before use. Check shelf date of acrylamide solution. 2. Be careful not to pierce wells while loading samples.

7.4 Constant Denaturant Gradient Gel

Problem	Solution
Fuzzy DNA bands	1. Clean wells prior to loading samples. 2. Use matching comb and spacer thickness. 3. Allow gel to polymerize for 60 minutes.
Smear at top of gel	1. Probably genomic DNA; this is OK.
Bands don't migrate far into gel	1. Check buffer concentration. Buffer concentration may be high. 2. Check acrylamide concentration. Acrylamide concentration may be too high. 3. Check gel solution concentrations. Make new gel solutions. Denaturant concentration may be too high. 4. Check voltage to DCode Universal Mutation Detection System. Voltage may be too low.
Streaking or DNA spikes in gel	1. Impurities in acrylamide. Filter before use. Check shelf life date of acrylamide solution. 2. Be careful not to pierce wells while loading samples.

7.5 Temporal Temperature Gradient Gel

Problem	Solution
Fuzzy DNA bands	1. Clean wells prior to loading samples. 2. Use matching comb and spacer thickness. 3. Allow gel to polymerize for 60 minutes.
Smear at top of gel	1. Probably genomic DNA, this is OK.
Bands don't migrate far into gel	1. Check buffer concentration. Buffer concentration may be high. 2. Check acrylamide concentration. Acrylamide concentration may be too high. 3. Check voltage to DCode System. Voltage may be too low. 4. Make sure the specified ramp rate parameter is used.
Streaking or DNA spikes in gel	1. Impurities in acrylamide. Filter before use. Check shelf life date of acrylamide solution. 2. Be careful not to pierce well while loading.
Bands did not resolve	1. Make sure the specified run parameters are used. 2. Check gel concentration.

Section 8
References

1. Fischer, S. G., and Lerman L. S., *Proc. Natl. Acad. Sci.*, **80**, 1579–1583 (1983).
2. Hovig, E., Smith-Sorensen, B., Brogger, A. and Børresen, A. L., *Mutat. Res.*, **263** (1), 63–71 (1991).
3. Sheffield, V. C., Cox, D. R., Lerman, L. S. and Myers, R. M., *Proc. Natl. Acad. Sci.*, **86** (1) 232-236 (1989).
4. Myers, R. M., Fischer, S. G., Lerman, L. S. and Maniatis, T., *Nucleic Acids Res.* **13** (9), 3131-3145 (1985).
5. Myers, R. M., Fischer, S. G., Maniatis, T. and Lerman, L. S., *Nucleic Acids Res.*, **13** (9), 3111-3129 (1985).
6. Børresen, A. L., Hovig, E., Smith-Sorensen, B., Malkin, D., Lystad, S., Andersen, T., Nesland, J., Isselbacher, K. J., and Friend, S., *Proc. Natl. Acad. Sci.*, **88**, 8405-8409 (1991).
7. Yoshino, K., Nishigaki, K., and Husimi, Y., *Nucleic Acids Res.*, **19**, 3153 (1991).
8. D'Aquila, R., Bechtel, L., Videler, J., Eron, J., Gorczyca, P. and Kaplan, J., *Nucleic Acids Res.*, **13**, 3749 (1991).

Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547
4100104 Rev B