

Laboratory 8

Restriction Analysis of Purified pARA-R

INTRODUCTION

The restriction analysis will provide final “proof” that the cells transformed during Laboratory 6, cloned overnight in LB/amp and purified in Lab 10 is the recombinant pARA-R. Two transformed colonies were selected for overnight culture in LB/amp broth, one is suspected to contain the pARA-R (**R**) and the other (**W**) is suspected to contain the *amp^r* gene but not the *rfp* gene. Although it is possible that the **W** plasmid has the *rfp* gene but is oriented in a way that does not allow its expression. The **W** colony was selected as a *negative* control to contrast the plasmid from cells that do expression the mFP protein.

You will be given as additional controls, an aliquot of known pARA-R to run as a *positive* control. This plasmid sample was cloned and isolated from LMG 194 using a commercially available plasmid purification kit (Qiagen). The recombinant plasmid that you’ve isolated from the **R** culture should produce an identical banding pattern to the plasmid used as the positive control.

The 5x *Hind* III restriction buffer you will use to digest these plasmids will contain *RNase*. The miniprep protocol you used to isolate plasmids from the **R** and **W** cultures is contaminated with RNA. If the RNA content of these miniprep plasmids is high, it is possible that it will obscure the banding of restriction fragments resulting from plasmid digestion. *RNase* will digest any RNA molecules into small fragments and these fragments will run far ahead of any of the plasmid fragments that we are interested in visualizing- i.e., the 702 bp *rfp* fragment.

MATERIALS

Reagents

pARA-R (40 ng/ μ L),
Miniprep **R** and **W** plasmids
1 Kb DNA ladder
Enzyme mix *Bam*H I + *Hind* III
5x restriction buffer/*RNase*
25 mL agarose (0.8%)
Distilled water
1x SB buffer
5x loading dye
Ethidium bromide (1 μ g/mL)

Equipment and supplies

P-20 micropipette and tips
1.5-mL microfuge tubes
37°C and 60°C water baths
Minicentrifuge
Electrophoresis apparatus
UV transilluminator
Video camera and thermal printer
Permanent marker
Crushed ice (optional)
Vortex apparatus

METHODS

This protocol will be similar to the protocol used to analyze pARA and pKAN-R but use the miniprep plasmids you isolated from the R and W overnight cultures. A known sample of pARA-R will be used as a positive control to compare restriction patterns that result from the miniprep plasmid digests.

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Preparing the restriction digest

1. Obtain a tube of pARA-R (positive control), **R** and **W** miniprep plasmids, 5x-restriction buffer/RNase and *Hind* III. Keep all three tubes in ice.
2. Use a marker to label six 1.5-mL microfuge tubes as follows:
 - A+** = pARA-R and enzyme mix
 - A-** = uncut pARA-R (no enzyme mix)
 - R+** = **R** from miniprep and enzyme mix
 - W+** = **W** from miniprep and enzyme mix
 - R-** = **R** from miniprep (no enzyme mix)
 - W-** = **W** from miniprep (no enzyme mix)
3. The following reaction matrix summarizes the restriction digest. Follow the steps beginning in step 4.

Tube	pARA-R	Miniprep DNA	Buffer/RNase	Enzyme	dH ₂ O	Total volume
A+	5 μ L	-	3 μ L	2 μ L	-	10 μ L
A-	5 μ L	-	3 μ L	-	2 μ L	10 μ L
R+	-	5 μ L	3 μ L	2 μ L	-	10 μ L
W+	-	5 μ L	3 μ L	2 μ L	-	10 μ L
R-	-	5 μ L	3 μ L	-	2 μ L	10 μ L
W-	-	5 μ L	3 μ L	-	2 μ L	10 μ L

4. Add 3 μ L 5x restriction buffer/RNase to the all six tubes.
5. Use a fresh tip and add the appropriate amount of distilled water to all six tubes.
6. Add 5 μ L of pARA-R to both **A** tubes.
7. Use a fresh tip and add 5 μ L of miniprep **R** DNA to tubes labeled **R+** and **R-**.
8. Use a fresh tip and add 5 μ L of miniprep **W** DNA to tubes labeled **W+** and **W-**.
9. Use a fresh tip and add 2 μ L *Hind* III, to *all* of the “+” tubes. Change tips to avoid contamination.
10. Cap the tubes, finger vortex to mix reagents and pulse centrifuge to spin down the sample.
11. Place all six tubes into the 37°C water bath, and incubate for approximately 60 **minutes**.

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Following the 30 minute incubation period, the digest can be frozen at -20°C until time is available for electrophoresis.

Electrophoresis of pARA-R restriction fragments

1. A 0.8% agarose solution has been pre-measured and is sitting in the hot water bath. Vortex to be certain that all agarose has been completely dissolved. No agarose lumps should be visible.
2. Prepare the gel tray for casting by seating the comb in the pair of slots nearest the end of the gel tray. Pour the liquid agarose into the gel tray without producing bubbles.
3. Allow the gel to solidify (approximately 20 minutes). Set the tray into the chamber so that the comb is nearest the negative electrode.
4. Fill the box with 1x SB buffer to a level that just covers the entire surface of the gel to a depth of 1-2 mm. Carefully remove the comb, taking care not to tear the wells. Add additional buffer if necessary.
5. Add 2 μL of loading dye to the tubes containing plasmids. Mix reagents by finger vortexing and spin in the minifuge to pool the reagents.
6. Obtain an aliquot of the 1 KB DNA ladder. This sample already contains loading dye.
7. Using a fresh tip for each sample, load **12 μL** of each plasmid sample and 10 μL of the 1 KB DNA ladder into the *center* seven wells in the order indicated:



8. Secure the cover to the electrophoresis box and connect the electrical leads to the power supply. Be certain that the anode is connected to the anode (red to red) and cathode is connected to the cathode (black to black).
9. Before turning the power supply “on,” rotate the voltage adjustment *counterclockwise* to set the voltage to zero. Turn “on” the power supply. Set the voltage to 130-135v. Electrophorese for **30-40 minutes**.
10. When the yellow dye has moved to within 2-3 cm from the far edge (+ end) of the gel, set the voltage back to zero, and turn the power supply off. Unplug the electrical leads from the power supply by grasping the plugs, not the cords. Remove the cover to the electrophoresis chamber.

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11. Gently slide the gel into the staining tray. Place a piece of tape on the cover the tray and put your name or group number on the tape.
12. Glove-up and cover the gel with ethidium bromide. NOTE: Ethidium bromide is a carcinogen and it is important to wear gloves when staining gels. Stain gels for 10-15 minutes.
13. Pour the ethidium bromide back into the container; it can be used several times. Cover the gel with deionized water (if available). Allow the gel to de-stain for 10-15 minutes. Pour the de-staining water into the sink and rinse the gel being careful not to let it slip out of the staining tray.
14. Use the photo-document your gel using the available equipment.

CONCLUSIONS

1. Review the restriction maps of the pARA and pKAN-R plasmids. Note the location of the *Bam*H I and *Hind* III restriction sites and size of the *rfp* fragment. Compare these resulting restriction fragments with the bands that appear in the 1-kb ladder lane. Do the fragments appear to be of correct size?



