

SSR Protocol

The plate you received contains 5 ug of DNA/well, enough for 100 PCR reactions per sample. Resuspend the DNA from the microtiter plate in 200 ul of sterile high quality (preferably 18 megaOhm) water per well to a final concentration of 25 ng/ul. This plate is now your master plate for all subsequent PCRs. Aliquot 2 ul per well from the master plate into a new thin-walled microtiter plate for PCR. DNA can be dried down after aliquot at 37°C and stored in the freezer until future use. If you choose not to aliquot all of the DNA from the master plate right away, store the unused DNA in the freezer

(-20°C). Polymerase chain reaction (PCR) conditions and cycling profile are based on the protocol established by E. Lynn Senior with slight modification mainly to accommodate the specific Taq polymerase used. Two reaction set ups are listed depending on whether you prefer to mix your own cocktail or purchase it premixed.

100 ul of each control primer pair is also included in the kit. The forward and reverse primers have been combined into the same tube to simplify set-up so that 1 ul of the control primer contains 50 ng of forward and 50 ng of reverse primer.

Reaction components (final concentration)

Mix your own cocktail:

1X PCR buffer

2.5 mM magnesium chloride

0.4 mM each dATP, dCTP, dGTP, dTTP

50 ng each forward and reverse primers (1 ul of control F/R primer mix)

0.3 units AmpliTaq Gold (Applied Biosystems) or Platinum Taq (Life Technologies, Bethesda, MD)

50 ng genomic DNA (2 ul of resuspended kit DNA)

sterile water to bring volume to 15 ul

If you prefer to buy a premixed cocktail you can purchase the RedTaq Jump Start redi-mix (Sigma). It contains all of the components except the primers and DNA. This mixture also contains a red dye so there is no need to add dye after amplification. The red dye does not interfere with PCR like the modified 5X SGB does. Instructions for set up with RedTaq are listed below.

-or-

RedTaq (1 reaction)

10 ul of 2X RedTaq

50 ng each of forward and reverse primer (1 ul of control primers)

50 ng of DNA (2 ul from the kit)

Sterile ultrapure water to bring volume to 19 ul.

Regardless of which of the above mixtures you use, we recommend making a bulk mix in a reagent basin containing all components but the template DNA that is enough for 100 reactions and then pipeting it into the plate containing your DNA using a multichannel pipet.

Thermocycling is carried out in a 96-well thin-walled microtiter style plate with an oil overlay. 13 ul of oil makes a drop that will come off the pipet tip without having to touch the components in the well. We have used this protocol successfully in an AmpliTron II (Barnstead-Thermolyne), a Tetrad (MJ Research), and a Mastercycler (Eppendorf).

Cycling Program

Preliminary dwell: 95°C 8-10 minutes (for AmpliTaq Gold only)*

One cycle of:

95°C 1 minute

65°C 1 minute

72°C 1 minute 30 seconds

then a one-degree decrement for the annealing temperature per cycle until the annealing temperature is 55°C.

Then 30 cycles of:

95°C for 1 minute

55°C for 1 minute

72°C for 1 minute 30 seconds

*When AmpliTaq Gold is used, the cycling profile should include a preliminary 8-10 minute dwell at 95°C to activate the AmpliTaq Gold. This is not needed if you are using Platinum Taq or RedTaq.

Making a gel to analyze your SSR amplification products

Super Fine Resolution (SFR) agarose (Amresco) is used to resolve the amplified bands. Metaphor agarose is an equivalent quality brand (FMC). A 4% SFR gel resolves fragments in the 50 –500 bp range. Make your gel in a 500 ml flask. Gel volume for the boxes discussed below is 150 ml. The first time a gel is made, follow the manufacturers recommendation for **slowly** adding the agarose powder to the **fast** swirling 1X TBE. After the agarose is thoroughly wetted it can be microwaved to melt the gel.

Microwave for 1 minute then hand swirl. Keep repeating this until it approaches the boiling point. The first time this agarose is melted, it tends to trap a lot of air and it boils over quickly so watch it carefully and open the microwave door as soon as you see any evidence it might boil. Gently swirl the flask after the boiling has subsided. After the gel has started to boil a couple of times, place it on the stir plate and let it stir for a minute. Then put it back into the microwave and heat to boiling again. Keep heating and stirring until the bubbles in the agarose go away (this may take awhile). After the gel is in solution make

a mark on the side of the flask with a sharpie to indicate the level of the agarose, stir bar plus the 1X TBE. This will insure the same volume and concentration each time you use this gel. Each flask should also have the date and id # on the glass and a piece of tape with the id # on it. You will need this mark and information when you remelt the gel for another use to replace volume lost by evaporation. Put the agarose solution back onto the stir plate for 10 minutes to cool. After 10 minutes, add ethidium bromide to 2 ug/ml (on the first use only). Pour the gel when the agarose is cool enough that you can comfortably hold the bottom of the flask to your palm. Be sure to transfer the tape with the ID # to the gel tray so you can remelt the gel in the correct flask. Make sure gel is level. Insert the 4 combs into the gel. Cover the gel immediately with plastic wrap to prevent it from cooling too fast. Do not move the gel until completely solidified. Remove the bumpers, wrap it and put it in the refrigerator for use the following day.

This agarose can be reused numerous times (30-50) and prior to repouring you will only need a little more ethidium (5-10 ul of a 10 mg/ml solution) after each remelt. (In between uses, the gels should be stored in a tub of 1X TBE. Be sure that the buffer covers all of the gels during storage.)

To remelt a gel. Match the corresponding gel and flask. Crumple the gel into the flask. Put the id tape onto the flask again. Remelt the gels. (This can be done in about 4.5 minutes on a heating stir plate.) Be sure that the gel is stirring during melting. After remelting, always check that the level makes the mark on the side of the flask, if not, add 1X TBE to volume. Loosely add a plug of kimwipe to the top to prevent evaporation. Put the flask into the microwave and heat again until boiling. Stir and cool. Add 10-15 ul of 10 mg/ml ethidium bromide solution and stir until ready to pour.

The only reason to stop reusing a gel is when an undesirable amount of background fluorescence builds up or when the concentration is obviously lower than the original concentration.

Gel boxes

The microtiter plate has been set up to load with a 12-channel pipet (titer plate layout sheet is included on this CD in a separate file). After PCR add 2 ul of modified 5X SGB dye to each sample prior to loading. Do not use regular 5X SGB dye as it will turn the entire gel blue for re-use. You can skip this step if you are using the premixed RedTaq solution. We have found that the Life Technologies Sunrise 96 gel boxes with the 26-well combs work well for SSRs. An entire microtiter plate can be loaded into one gel with this configuration. We leave the outer two spaces cast by the 26-well comb for loading molecular weight marker (wells 1 and 26). (If your population requires multiple microtiter plates to represent all the individuals, it is a good idea for tracking purposes, to load a molecular weight marker on different tiers of the gel for each master plate.) For example, load a marker on the first tier to indicate the first titer plate, load on the second tier for the second plate of the population. The molecular weight marker should have fragments every 100 bp, starting at 50-100 bp. Because of the high percentage agarose, fragments over 1000 bp will not separate.

To run the gel, fill the gel box with 1X TBE but do not cover the center bridge. Unwrap the gels and place them into the gel box. Remove the combs. Place the PCR plate on the counter at the bottom of the gel box and carefully remove the lid. The 12-channel pipettor is spaced such that it loads every other well of the 26-well comb. We begin by drawing up 18 ul per well from the samples in row A in the microtiter plate and loading it starting with space number 2 on the top comb. This will load spaces 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 of the first comb. Be careful to avoid drawing up the mineral oil as it makes the DNA samples float out of the wells. Next we draw up row B from the microtiter plate and load spaces 3,

5, 7, 9, 11, 13, 15, 17, 19, 21, 23, and 25 of the top comb. Row C begins in space 2 of tier 2, row D in space 3 of tier two, etc. (See table below). Loading in this way will result in consecutive order of the sample across tier 1, then tier 2, 3, and 4. Load 15 ul of 100 bp molecular weight marker to the appropriate lane. Add 1-2 drops of 10 mg/ml ethidium bromide to the buffer solution in the lower part of the gel box.

After all the samples are loaded slowly add more 1X TBE to the lower buffer chamber on the gel box until it completely covers the gel. If you rush this step, your samples will come out of the wells and you'll have to start over again. Cover the gel as deeply as possible. The deeper the buffer the longer the gel can run before depleting the buffering capacity.

Loading orientation from microtiter plate to gel:

Row in Microtiter Plate	Starting Space	Tier
A	2	1
B	3	1
C	2	2
D	3	2
E	2	3
F	3	3
G	2	4
H	3	4

5X TBE

270 g Tris Base

137.5 g boric acid

23.25 g disodium EDTA

Stir to dissolve and bring to 5 liters.

pH should be around 8.3 without adjustment.

1X TBE from 5X TBE

(make in a 20 L carboy)

Fill carboy with about 15 liters of ultra pure water. Add 4 liters of 5X TBE. Bring volume to 20L with ultrapure water. Tighten cap and mix well by tilting side to side several times.

10 mg/ml Ethidium Bromide

Dissolve 100 mg of ethidium bromide in 10 ml of dH₂O. Wrap tube in aluminum foil and store at 4°C.

Caution: Ethidium bromide is extremely mutagenic. Wear gloves, eye protection, and a lab coat when working with it.

5XSGB(10 ml final volume)

4.0 ml	ddH ₂ O
0.5 ml	1 M Tris pH 8.0
5.0 ml	Glycerol
100 ul	0.5 M EDTA pH 8.0
250 ul	20% SDS
1.5 mg	Bromphenol Blue Sodium Salt

Store in refrigerator or freezer.

Parental DNAs

The kit contains two additional tubes of 10 ug each of B73 and Mo17 parental DNAs. These are intended to be used for screening your primers for polymorphism prior to mapping. There is enough DNA for 200 screenings. It is not necessary to use this DNA to screen the control primers as we have already tested them for polymorphism. It is not necessary to add DNA from these tubes to the microtiter plate with the 94 lines as we have already included the parents in the plate.

To use the parental DNA for screening, resuspend each sample with 400 ul of sterile ultrapure water to a final concentration of 25 ng/ul. Store the DNA at -20°C except when you are using it.

Running the control primers

Before mapping your gene(s) of interest, you will need to run PCR amplification and gels for the three control primers included in the kit. **Do not proceed with mapping any other primer pairs** until an exact match is obtained between the control primer scores included in a separate file on this CD and your control primer scores.

Once you have run the control primers on a gel, score them according to the scheme given in the next section into the "control primer scores" file on this disk in the rows where it says "your scores con. 1", "your scores con. 2", and "your scores con. 3". Have some else double-check your scoring. After you are both satisfied with your scores, compare them to the row above containing our scores from the master plate with each of the primers. If you do not get a perfect match, recheck your gels to see if the mismatched individuals are miscoded. If there are still discrepancies between our scores and yours, email the "control primer scores" file with both your scores and ours plus the gel images for your controls as

attachments to IBMkits@missouri.edu. Please do not send the files in the body of your email message. We will look at your data as soon as possible and let you know how to proceed. If your gel images are not electronic, please email the scores file to the address above and FAX a copy of the images to 573-884-1469 attention: IBM Kits Coordinator. If you FAX please include your name, postal and email addresses so we can reply back. Please be patient as our staff donate their time to replicate plates and deal with questions and concerns, we will reply within a few days. The kit fees are used to pay for materials and student labor to extract DNA and process orders. They would be substantially higher if we had a full-time staff salary to support.

Scoring your mapping gels

The fragment generated by the B73 parent will always be scored as an A. Likewise, the fragment generated by the Mo17 parent will always be scored as a B. Scores for other possibilities are given below. Please use these conventions for consistency. You will note that there are several additional characters included that can be used to track sample or seed contamination. It is our experience that these can be really useful in identifying problems. We urge you to use them. Most mapping softwares do recognize K, Y, Z, or X as valid scores so these will need to be converted to appropriate characters by search and replace prior to mapping.

Score	Fragment
A	Fragment from parent A
B	Fragment from parent B
H	Fragment from parent A and parent B
-	Can't be scored
K	Fragment isn't from either parent
Y	Fragment from parent A plus a non-parental fragment
Z	Fragment from parent B plus a non-parental fragment
X	Fragments from both parent A and B plus a non-parental fragment
C	Could be either B or H
D	Could be either A or H