A Rieseberg Lab Production

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GBS

(Pronounced "jibs")

(Genotyping-By-Sequencing)

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Perfectors and Writers:

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REQUIRED REAGENTS

Reagent	Amount per 96 well plate
• 96 barcoded adapters (0.4ng/ul)	4.5ul of each
• Common adapters (0.4 ng/ul)	450ul
• Restriction enzyme PstI Hi-Fidelity	50ul
• NEB Buffer 4 (10X)	250ul
• BSA (10ug/ul)	50ul
• NEB T4 DNA Ligase (M0202L) – *NOTE: Do not purchase 'small' size as this is not enough for one plate.*	80ul
• NEB T4 DNA Ligase Buffer (10X)	500ul
• Qiaquick PCR purification kit	5-12 columns
• Taq 2x Master Mix (NEB, M0270L)	25-200ul

- GBS PCR primers A and B (12.5ug/ul each) — 8 ul each

- Bio-Rad Certified Low-Range Agarose -On the chemical shelf — 0.5 - 1.5 g

- 1KB Plus Fermentas DNA Ladder - Freezer — 10 ul

- 6X Frementas Loading Dye -Freezer — 20 ul

- SYBR gold nucleic acid gel stain (10000X) -Freezer — 5 - 15ul

- Zymoclean Gel DNA recovery kit — 1 - 3 columns

- Qubit 2.0 Fluorometer high-sens buffer and tubes — 1-3 reactions

- Hi-Sens DNA Bioanalyzer chip kit — 1 chip per 11 samples

- KAPA Library Quant Kit — variable

Notes before starting:

1. The DNA you intend on using in this protocol must be QCd using both the Nanodrop to check purity and the Qubit to assess quantity. It must be at least 8.5 ng/ul or may be under represented in your data.

2. Previous protocols used 50 ng of DNA per sample while we use 100 ng. Others have had success with 50 ng using CTAB extracted DNA, while we used Qiagen column extracted DNA. It is possible that CTAB extracted DNA works better for GBS. Regardless, more DNA seems to be better

3. Many of these steps can be done using the Aitken EpMotion, they are specified with the robot icon.  See additional instructions on using the robot. If you want to use the robot, speak to Kristin about it.

PROTOCOL

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1.  Plates of barcoded adaptors need to be prepared. To do this, transfer 4.5 uL of each barcoded adaptor from the master plate into a new plate. It is good practice to do a bunch of these at one time to minimize the number of times that the master plate needs to be thawed.

2.  Add 100 ng of DNA into the adaptor plate in a maximum of 11.7 uL water. If less than 11.7 uL is needed to add the full amount of DNA, add water to equal 11.7 uL. This is an ideal step to be done using the EpMotion. See 'Normalizing a Plate with the EpMotion' for detailed instructions.

3.  Create a digestion/ligation master mix and add 13.8 ul to each well (EpMotion works for dispensing master mix).

Reagent	X1 sample	X100 sample
Buffer 4	2.5ul	250ul
PstI Hi-Fi	0.5ul	50ul
BSA	0.5ul	50ul
T4 Ligase	0.8ul	80ul
Ligase Buffer	5ul	500ul
Common Adapter	4.5ul	450ul

4. Incubate in the thermal cycler using the following protocol:

Temperature	Time
37°C	3 hours
22°C	3 hours
65°C	20 minutes
4°C	forever

5.  After thermal cycling, remove 10 uL from each well into a single 2.0 mL tube. Mix the contents and divide equally into four 2.0 mL tubes (240 uL per tube). You must do this to accommodate the Qiaquick PCR purification buffer.

6. Clean up the four tubes using the Qiagen Qiaquick PCR purification kit. Run the contents of all four tubes though a single column to further concentrate the sample and elute in 50 uL TE buffer.

   - ***NOTE: DO NOT FREEZE THIS UNTIL YOU HAVE A COMPLETED LIBRARY. Greg and Kristin found that the PCR amplification does not work well using previously frozen template. We are not sure why this could be.***

7. Perform PCR amplification (x 8 reactions). Each reaction will have a total volume of 50 uL

Reagent	Volume x1	Volume x 8.15
H_2O	21ul	171.2ul
NEB 2x Taq Master Mix	25ul	203.8ul
GBS PCR primers A and B	1ul each	8.2ul each
Template from step #6	2ul	16.3ul

8. Cycle reactions using the following protocol:

Step	Temperature	Time
1	94°C	3 min
2	94°C	30 sec
3	65°C	30 sec
4	68°C	30 sec
5	Go to #2 17 more times	–
6	68°C	3 min
7	4°C	forever

9. Clean your PCR reactions using the Qiaquick kit. Run four of the reactions through one column and the other four through another column and elute each column with 40 ul of H_2O.

    - NOTE: This step is to concentrate the 400 ul of PCR product into 80 ul. We are experimenting with using a SpeedVac or Ampure beads as alternatives to increase the yield.

10. Pour a 2.0% gel using the Certified Low-Range Agarose found on the chemical shelf in a yellow container in the non-Ethidium bromide contaminated room. This gel is better at separating low bands than the normal agarose. You can run two libraries on the smaller blue gel rig, and possibly three in the larger orange one. We recommend using the same gel rig, % gel and voltage for all libraries.

11. Add 8 uL of 6x loading dye (found in the freezer) to each of your 40 ul cleaned PCR reactions. Mix 10 uL of 100 bp ladder with 2 uL 6x loading dye (This ladder seems extra weak). Load your gel, including both cleaned PCR reactions in separate neighbouring lanes, alternating between DNA and ladder, and run @ 100 V for 1.5 hours. Try to separate different libraries as much as possible.

12. Remove gel and soak in SYBR Gold solution for 30 minutes (Dilute the SYBR Gold 10000X in TAE, enough to cover the gel). Stain on a shaking table, protected from light. Do not de-stain.

13. Put the gel on the blue light box to visualize the bands.

14. The trick to GBS is to cut out the same size bands with each plate in order to maximize shared sites between plates. Cut the following slices using a razor or gel plucker.

Size	Range
S	350 - 450 bp
M (money slice)	450 - 550 bp
L	550 - 650 bp

15. Purify gel slices following the Zymo gel purification protocol. You will have two S fragments, two M fragments, and two L fragments. Melt gel in 2 ml tubes with prescribed solution at 60°C for 5-10 min. Run slices of the same size through one column to increase total amount of library and elute in 20 uL.

16. Perform QC using the Bioanalyzer to assess fragment size range, the Qubit to assess concentration of library and qPCR to check that adaptors have been correctly ligated. Only sequence your library if it passes all three QC tests! Ideally, the Qubit, Bioanalyzer and qPCR should give the same concentration readings (corrected for size of insert), although qPCR is most pertinent for sequencing. The quality required for sequencing in Biodiversity are:

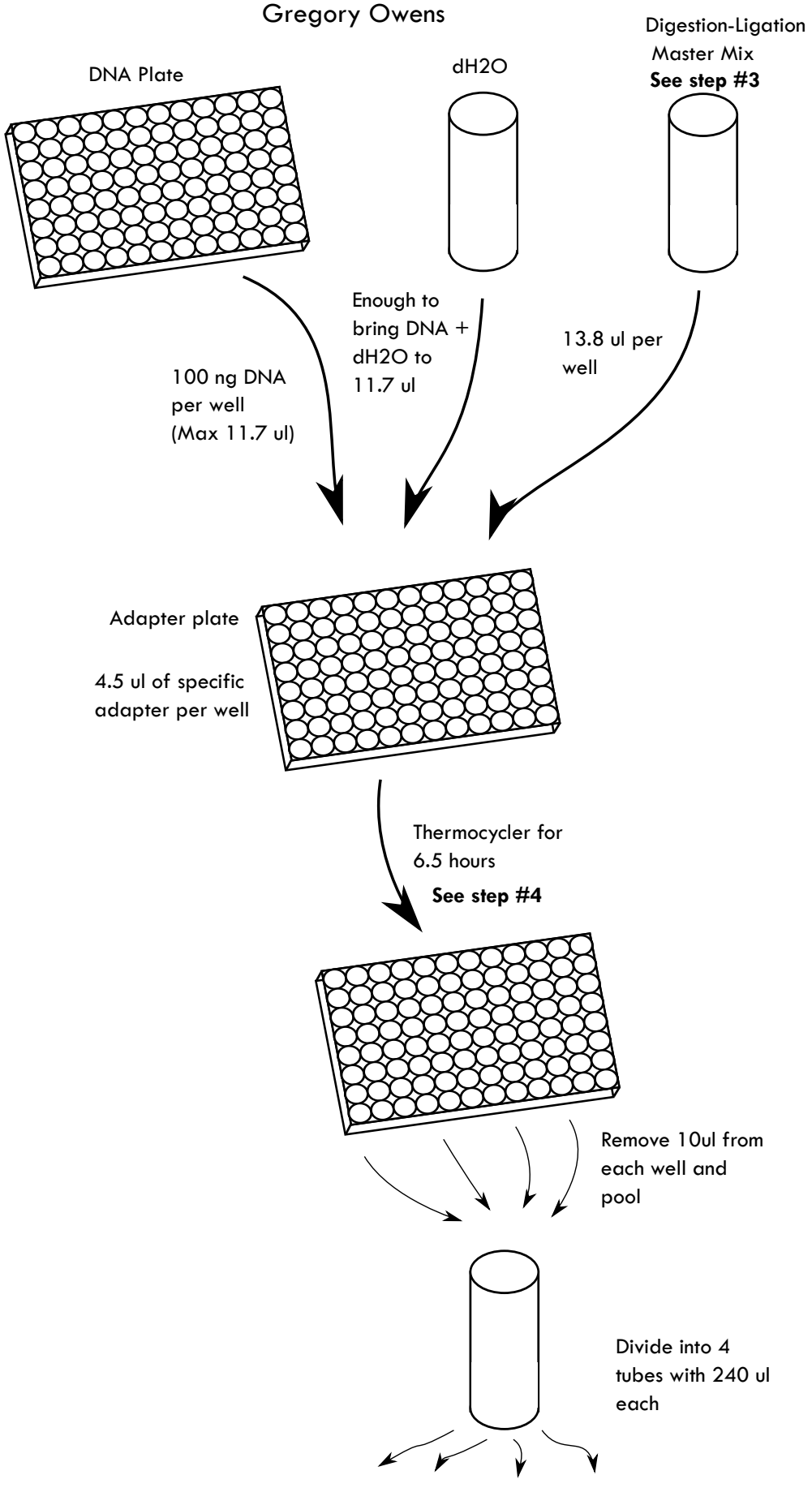
    - qPCR: Concentration should be greater than 2nM. Remember to take into account the average size of your library

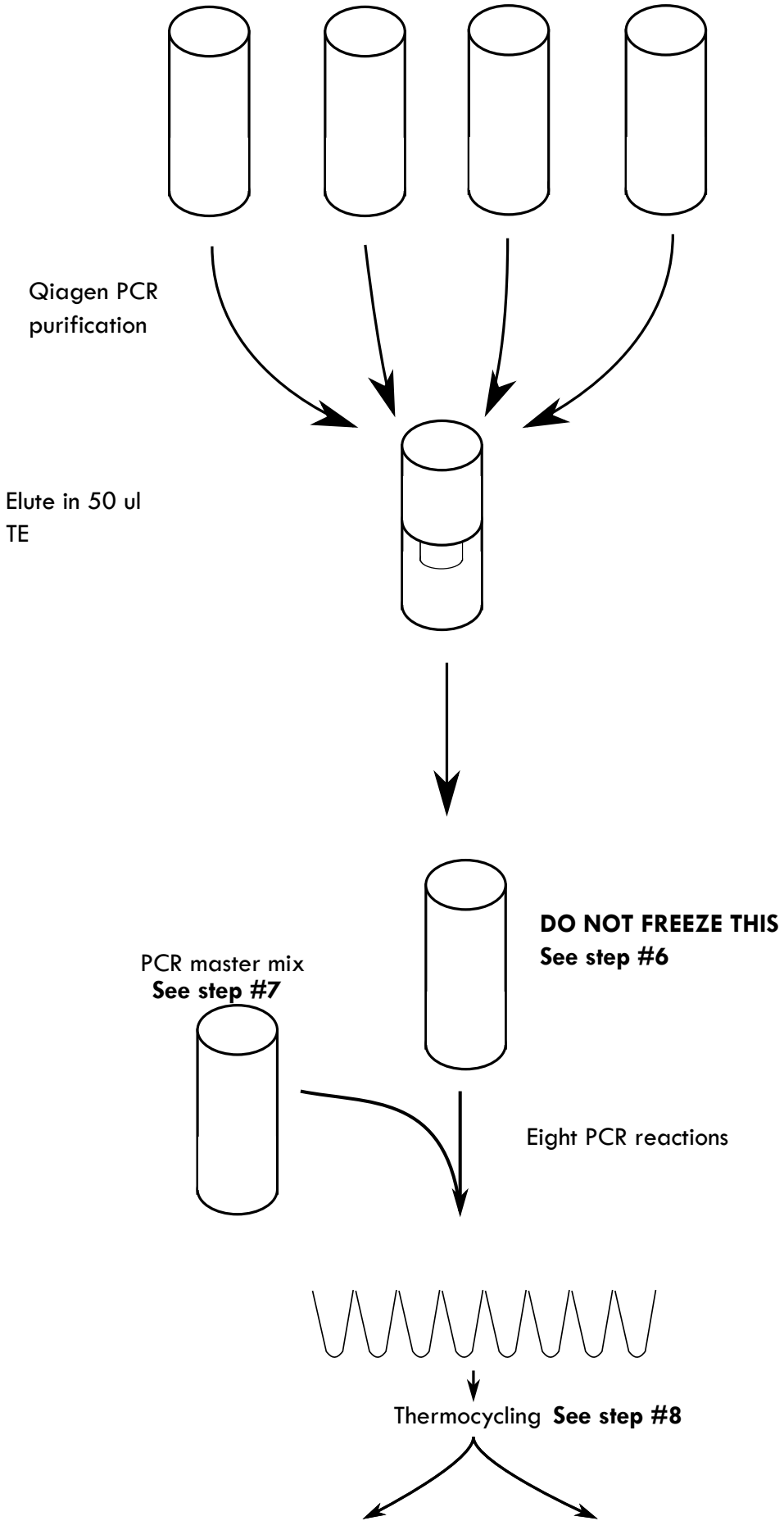
- Qubit: Greater than 0.5 ng/ul of total double stranded DNA.
- Bioanalyzer: There should be a single peak approximately 100 bp wide. It should be relatively smooth. Peak height is not necessarily quantitative but as a rough estimate your peak should be great than 50 FU in height.

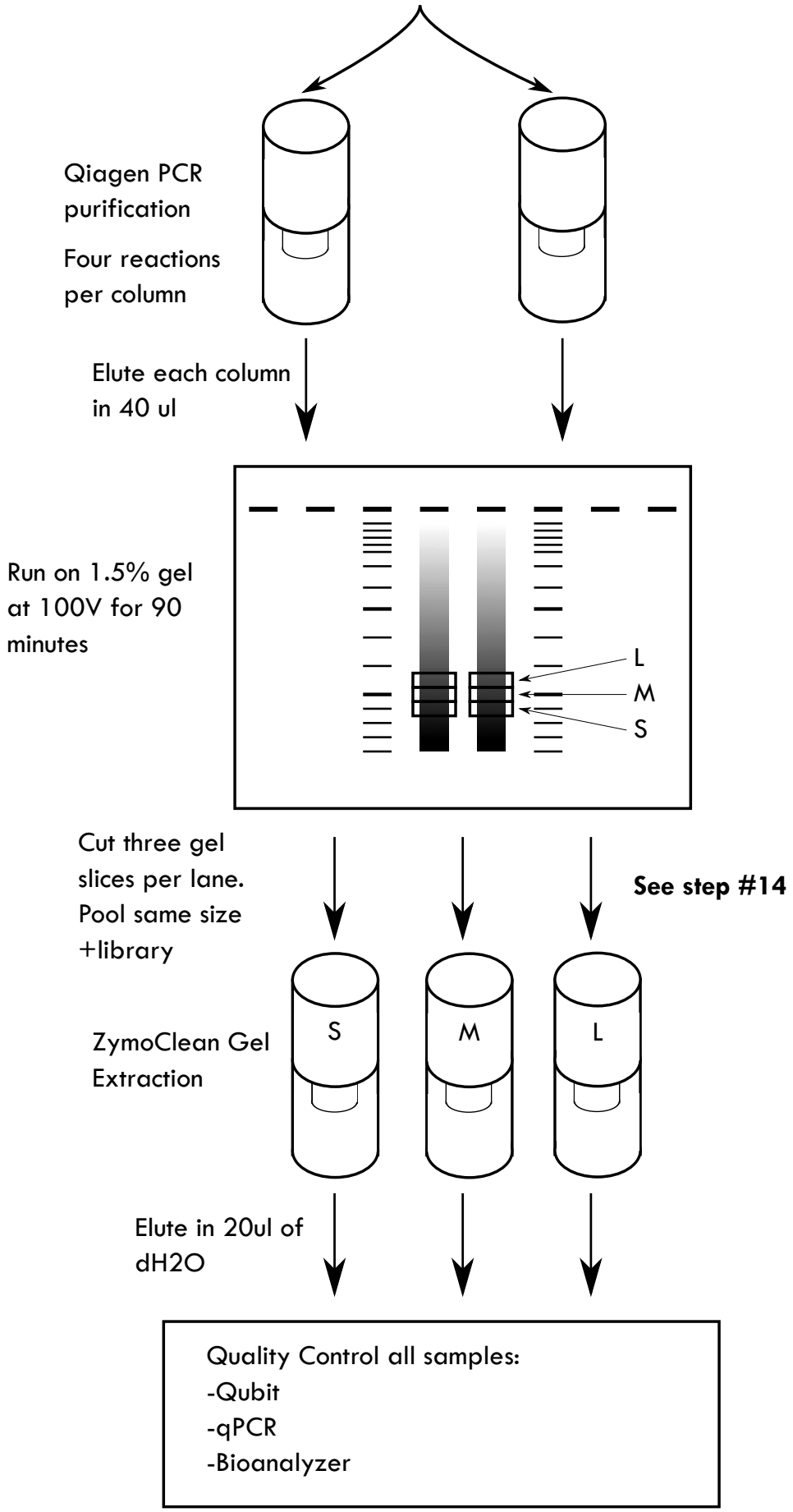
As the Qubit is the cheapest and easiest test, generally you should start with that and not continue if your Qubit result is much lower than the requirement

GBS Protocol Diagram

By Kristin Nurkowski and
Gregory Owens




Qiagen PCR
purification
Elute in 50 ul
TE
DO NOT FREEZE THIS
See step #6
PCR master mix
See step #7
Eight PCR reactions
Thermocycling See step #8


Qiagen PCR purification
Four reactions per column
Elute each column in 40 ul
Run on 1.5% gel at 100V for 90 minutes
L
M
S
Cut three gel slices per lane. Pool same size +library
See step #14
ZymoClean Gel Extraction
S
M
L
Elute in 20ul of dH2O
Quality Control all samples:
-Qubit
-qPCR
-Bioanalyzer