Langerhans Lab Protocols

NC STATE UNIVERSITY

To prepare samples for Genewiz DNA sequencing

GENEWIZ Research Triangle Park: Open 2:00 p.m. – 10:00 p.m. (ET) (+1-919-313-9682)

DNA Sequencing Services: http://www.genewiz.com/public/DNA-sequencing-services.aspx

DNA Sequencing Sample Submission Guidelines: http://www.genewiz.com/public/Sample-Submission-Guideline.aspx

STEPS:

1. **Amplify target region by PCR.**
2. **Run PCR product on agarose gel** to verify amplification and to check for non-specific products (i.e., extra bands).
3. **Purify PCR product** by gel extraction (if extra bands are present) or by using ExoSAP-IT.

   Gel extraction: use kit, usually Omega Bio-tek, located in bottom drawer to left of PCR hood in 382 DCL.

   ExoSAP-IT: follow steps in "Preparing PCR Product for Genewiz Sequencing" below.

   Genewiz says:

   PCR products should be purified by either gel extraction and eluted in water, or by enzymatic treatment. Please use gel extraction if you have more than one product from a PCR reaction (i.e. you have more than one band).

   If you have a single PCR band and would like GENEWIZ to perform the PCR clean-up step, simply order "Custom" service and choose "PCR Clean-up" from the "Special Request" column. Send the same amount of DNA that is required for purified PCR products. Also include a gel image of your PCR product with the volume loaded in each well clearly labeled, and the volume and mass of the ladder.

   If you would like to perform the PCR clean-up, we recommend the ExoSAP-IT kit from USB Corporation (Cat. #78200).

4. **Dilute PCR product** as necessary to achieve concentration requested by Genewiz -- see "Preparing PCR Product for Genewiz Sequencing" below.
5. **Label tubes** to be submitted to Genewiz per their protocol (see image):

   Genewiz says:

   If you are submitting <48 samples, please use 8-strip, 0.2 ml PCR tubes and caps. You can cut off empty tubes and use them next time.

   Label the tubes as shown:

   If you are submitting 48 or more samples, we recommend using a 96-well, semi-skirted PCR plate. Cap the wells with 8-strip caps. These are usually ordered separately from the plates. Be sure that the caps seal tightly! The semi-skirted plate helps to prevent the plate from bending in transit, resulting in fewer loose caps. Please ship the plate with cushioning to avoid potential damage.

Arrange your samples vertically (in columns) as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	control
H	8	16	24	32	40	48	56	64	72	80	88	control

6. **Fill tubes.** See **Preparing PCR Product for Genewiz Sequencing** below.
7. **Submit order through Genewiz web site.**
    a. each technician should start a new account, because Genewiz gives free sequencing coupon
    b. Langerhans lab usually uses Pre-Mixed samples (i.e., primer and sample are mixed in the tubes we send).
    c. special handling instructions: for ND2, choose “difficult template” for better results
    d. put tubes in a small bag (ex. ziplock sandwich bag, or other re-used clean bag), and label bag with bright tape that says “for Genewiz pickup”
    e. print a hard copy of the receipt to put in bag with tubes, and save a copy on your computer
    f. Genewiz says: “Please include your order receipt with your samples. Call us at 877-436-3949 if you have any questions and refer to your order tracking number.”
8. **Put samples in pick up location.**
    a. D. Clark Labs mail room is closest. Genewiz picks up at 3:30 pm.

Preparing PCR Product for Genewiz Sequencing

Remember that for sequencing, each reaction tube has PCR product (purified by ExoSAP-IT or gel extraction) and one primer. If forward and reverse sequence are needed, two reaction tubes must be submitted (one with PCR product + Forward primer, one with PCR product + Reverse primer). For all sites except CytB, one tube is needed for forward primer and a separate tube is used for reverse primer. With CytB, the sequence is short enough to use only Fwd primer.

To fill reaction tubes for Genewiz sequencing:

1. Determine how much DNA you need to submit per tube. See Genewiz web page [http://www.genewiz.com/public/Sample-Submission-Guideline.aspx]. Amount is based on fragment size.

   Genewiz’s Tips on How to Determine the Template Concentration for DNA Sequencing:

   *For **purified PCR products**, multiply 2 ng/µl by the length of the template in kilobases.*
   Optimal purified PCR product concentration = 2 ng/µl x kb
   Example: You have a 700 bp PCR product. Multiply 2 times the product size in kb. Thus, the optimal concentration for sequencing is 1.4 ng/µl. Since we request template in 10 µl, you would submit 14 ng purified PCR product. (see table below)

*In the **same tube**, mix template (10 µl) and your primer (5 µl) according to the table below:*

DNA Type	DNA Length (include vector)	Template Concentration in 10 µl	Template Total Mass	Your Primer Total Picomoles	Premixed Volume* (Template + Your Primer)
Purified PCR Products	<500 bp	~1 ng / µl	~10 ng	25 pmol	15 µl
	500 - 1000 bp	~2 ng / µl	~20 ng		
	1000 - 2000 bp	~4 ng / µl	~40 ng		
	2000 - 4000 bp	~6 ng / µl	~60 ng		
	>4000 bp	Treat as plasmid	Treat as plasmid		

DNA needed = **a** ng

2. Estimate the concentration of your PCR product from the band intensity on your gel, as compared to the ladder's intensity.

   For New England BioLabs's Quick-Load 100 bp DNA ladder, the upper bright band (1000 bp) is 9.5 ng/µl, diluted by how much loading dye + SYBR green you added

   ex.: 5 µl ladder + 1.5 µl [loading dye + SYBR green] = 7.3 ng/µl

   ex.: 3.5 µl ladder + 1.5 µl [loading dye + SYBR green] = 6.6 ng/µl

   estimate concentration of band on gel = **b** ng/µl

   calculate the concentration of your PCR product before it was diluted with loading dye:

   PCR product conc. = **c** ng/µl = **b** ng/ µl x total vol. loaded ÷ vol. of PCR product loaded

   ex.: **c** ng/µl = **b** ng/µl x 6.5 µl ÷ 5 µl when 5 µl sample + 1.5 µl [loading dye + SYBR green] used

   see Protocol for Low Concentrations below for very faint bands, where concentration (**c**) <= 3.2 ng/µl

3. Determine how much PCR product to use in the ExoSAP-IT reaction tube:

   for forward and reverse sequencing (i.e., 2 tubes), you need **a** ng x 2

   vol of PCR product you need = **d** µl = 2**a** ÷ **c**

4. Calculate how much ExoSAP-IT to use per reaction tube:

   use 2 µl ExoSAP-IT per 5 µl PCR product

   amt to use = **e** µl = **d** µl x 2 ÷ 5

5. Treat PCR product with ExoSAP-IT:

   Keep ExoSAP-It and tubes containing it on ice.

   Prepare one PCR tube / sample (not one per primer per sample).

   Mix **d** µl of PCR product + **e** µl of ExoSAP-IT in new PCR tubes.

   Incubate in thermocycler per ExoSAP-IT instructions: 37°C for 15 minutes, then 80°C for 15 minutes.

6. Calculate the post-ExoSAP-IT PCR product concentration:

   (**d** µl x **c** ng/µl) ÷ (**e** µl + **d** µl) = **f** ng/µl = final concentration of PCR product in ExoSAP-IT tube

7. Calculate how much post-ExoSAP-IT PCR product to put in Genewiz tube (= **g** µl). Note that Genewiz wants a final tube vol of 15 µl, including primer. Langerhans lab primer concentration is usually 10 µM and Genewiz wants 5 µl of 5 µM (= 25 mol), so add 2.5 µl of 10 µM of one primer per reaction tube. Thus the maximum post-ExoSAP-IT PCR product you can add to the reaction tube is 12.5 µl.

   g µl = **a** ng ÷ **f** ng/µl where **g** µl <= 12.5 µl

8. To fill tubes, add per tube:
   - enough molecular grade water to make final vol = 15 µl

     vol. water to add = 15 µl – **g** µl – 2.5 µl
   - 2.5 µl of Forward OR Reverse 10 µM primer
   - **g** µl post-ExoSAP-IT PCR product

Protocol for Low Concentrations (for very faint bands)

Use this protocol when your estimated PCR product concentration is < 3.2 ng/µl (when 40 ng needed rxn tube; when other amount needed, this cut off will change)

to maximize the amount of DNA per Genewiz tube:

- mix 18 µL PCR product + 7.2 µL ExoSAP-IT per tube; heat per ExoSAP-IT instructions
- then mix into sequencing tube: 2.5 µL of 10 µM F or R primer + 12.5 µL post-ExoSAP-IT product

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Calculations table for preparing samples for Genewiz sequencing

date	**sample #**	**PCR date/tube #**	**a** ng	**b** ng/µl	**c** ng/µl	**d** µl	**e** µl	**f** ng/µl	**g** µl