Host-Microbiome Research Network Germ-free mouse monitoring protocol

Environmental monitoring for microbial contamination is included in per diem charges in the GF facility.

Step 1: Routine Monitoring
Food, water, bedding, and stool pellet from each cage are collected every 2 weeks (at cage change) into sterile nutrient broth. These samples and positive controls from the SPF facility (stool pellet and bedding) are sub-cultured after 24 hours into sterile LB and Sabouraud dextrose broths and incubated (without shaking) at 37^{o}C for 48 hours. At this time they are visually examined against the positive and negative (sterile water) controls and any turbidity is ranked + (low), ++ (medium) or +++ (high). Samples with low turbidity are considered negative for culture.

Step 2: Follow-up Monitoring
In the case of samples with medium or high turbidity (i.e. with comparable turbidity to the SPF samples in LB media), follow-up anaerobic culturing is then performed. In the anaerobic chamber, stool samples in the original nutrient broth are inoculated into modified yeast-starch-glucose broth and cooked meat clostridia media for more fastidious anaerobes. Turbidity is monitored over 2 weeks and ranked as previously described; lack of turbidity after this time indicates a negative result.

In parallel, qPCR analysis is performed on stool pellets using bacterial and/ or fungal primers. Total DNA is extracted using the NucleoSpin® Soil kit (Macherey-Nagel) following manufacturer's instructions for high yield and eluted in a 40 µl volume. Typically, SPF pellets have >100 ng/µl DNA whereas pellets from GF mice have <10 ng/µl. All samples are diluted 10-fold and run in triplicate on the CFX384 Real-time system (Biorad) along with a negative (water) control. Each 10 µl reaction consists of 4 µl diluted sample, 0.5 µl each of forward and reverse primers (5 µM) and 5 µl sybr green. See table of primers and annealing temperatures below. The qPCR reaction includes an initial denaturation at 95^{o}C, followed by 40 cycles of 95^{o}C (30 s), 52-60^{o}C (30 s) and 72 (1 min), with generation of a dissociation curve (55-95^{o}C) to verify amplification specificity.

Table 1. List of primers for microbial DNA qPCR analysis.

Target Group	Primer	Sequence (5'-3')	Tm
Bacterial 16S rRNA	UniF340	ACT CCT ACG GGA GGC AGC AGT	62.6
	UniR514	ATT ACC GCG GCT GCT GGC	61.7
Bacterial RNA polymerase B	rpoB1698f	AAC ATC GGT TTG ATC AAC	47.7
	rpoB2041r	CGT TGC ATG TTG GTA CCC AT	55.8
Fungal ITS (18S small subunit- 5.8S)	ITS1F	CTT GGT CAT TTA GAG GAA GTA A	49.7

	ITS2	GCT GCG TTC TTC ATC GAT GC	57
Fungal ITS (28S large subunit- 5.8S)	ITS4	TCC TCC GCT TAT TGA TAT GC	52.1
	ITS86F	GTG AAT CAT CGA ATC TTT GAA	48.6

Typically, the positive controls for the 16S primers have CT values of ~10-12 while negative controls show CT values >30. Test samples are considered negative if they are 15 or more CTs (i.e. DNA doubling reactions) greater than positive controls.

Step 3: Those samples that are positive by anaerobic culturing and/or with positive 16S results (i.e. with similar CTs to SPF) are considered positive for bacterial contamination. In this event, the PI will be contacted and sacrificing animals will be recommended. If desired, testing a subsequent cage change can be considered at additional cost of tech time to the PI.

Further Reading:

Hecht G, Bar-Nathan, C, Milite G, Alon I, Moshe Y, Greenfeld L, Dotsenko N, Suez J, Levy M, Thaiss CA, Dafni H, Elinav E and Harmelin A. 2014. A simple cage-autonomous method for the maintenance of the barrier status of germ-free mice during experimentation. Laboratory Aminals 48(4): 292-297.