Steroid Extraction Protocol – Fecal Samples

INTRODUCTION

This protocol serves as a general guideline for extracting steroids from fecal samples and determining extraction efficiency. Some steps may require adjustment depending on your sample and experimental setup. Always validate your extraction procedure before applying it to high-value samples.

MATERIALS AND EQUIPMENT NEEDED

- Ethanol (≥ 95% purity)
- Test tubes capable of holding ≥ 1 mL
- Centrifuge capable of 5,000 rpm and an incubation temperature of 4°C
- DetectX® kit-specific 1X Assay Buffer
- DetectX® kit-specific Steroid standard for extraction efficiency determination
- Orbital shaker with test tube adapter
- Analytical balance
- Grinding equipment (mortar and pestle, mixer mill, etc.)
- Fume hood
- Centrifugal vacuum device (optional)
- Oven (optional)
- Lyophilizer (optional)
- Parafilm (optional)

PROCEDURE

Ensure that the sample is completely dry. Samples can be dried passively in a clean and protected fume hood, via gentle heating (≤ 60°C), or by freeze-drying (lyophilization). Grind the dry sample into a powder using the appropriate grinding equipment to improve extraction recovery. Remove any large particles not containing the steroid of interest (e.g., grass) when possible. To obtain a more accurate reading of the analyte concentration of the sample, the extraction efficiency must be determined. Refer to the Extraction Efficiency section below for details.

1. Weigh out ≥ 0.2 g of dried, powdered sample into a test tube.
2. Add 1 mL of ethanol for every 0.1 g of sample. Cap the tube or seal with parafilm.
3. Shake sample using an orbital shaker capable of holding test tubes for at least 30 minutes.
4. Centrifuge sample at 5,000 rpm for 15 minutes at 4°C. Transfer the analyte-containing supernatant to a clean tube. This supernatant can be stored at ≤ -20°C for one month.

   Note: Samples containing low levels of analyte can be concentrated by evaporating the supernatant completely using a centrifugal vacuum device and resuspending in a reduced volume of 1X Assay Buffer.
5. **These are general guidelines. It is important to verify the maximum ethanol tolerance of each assay as described in the "Interference" section in the assay kit insert. Volumes may need to be adjusted so the sample falls within the standard curve of the assay and adheres to the ethanol tolerance of the assay.** Add 100 µL of supernatant from step 4 into a minimum volume of 400 µL 1X Assay Buffer (Supernatant Volume + 1X Assay Buffer Volume = Total Reconstitution Volume). Briefly vortex and incubate the sample for 5 minutes at room temperature. Repeat vortex and incubation three times to ensure complete steroid solubility. Continue to dilute the sample in 1X Assay Buffer as needed (Assay Dilution Factor).
6. Run samples in the assay immediately according to the assay protocol to obtain sample concentrations (Assay Concentration).

EXTRACTION EFFICIENCY

To determine extraction efficiency, one powdered sample will undergo the extraction process twice; once with a known amount of analyte added (Spiked Control) and once with an equivalent volume of 1X Assay Buffer added (Unspiked Control). A third control (Assay Buffer Control) must also be prepared but does not undergo the extraction process. All aliquots must have equivalent volumes (V1) and all spikes must have equivalent volumes (V2). The preparation of these controls is summarized in the table and figure below.

The three controls are then run in the assay according to the assay protocol. Extraction efficiency is calculated using the following formula:

$$\text{Extraction Efficiency (\%)} = \frac{(\text{Spike Control Concentration} - \text{Unspiked Control Concentration})}{\text{Assay Buffer Control Concentration}} \times 100$$

Extraction Condition	Spiked Control	Unspiked Control	Assay Buffer Control*
Aliquot (V1)	Sample	Sample	1X Assay Buffer
Spike (V2)	Steroid Standard	1X Assay Buffer	Steroid Standard

*The Assay Buffer Control does not undergo the extraction process. Prepare this control immediately prior to assaying samples.

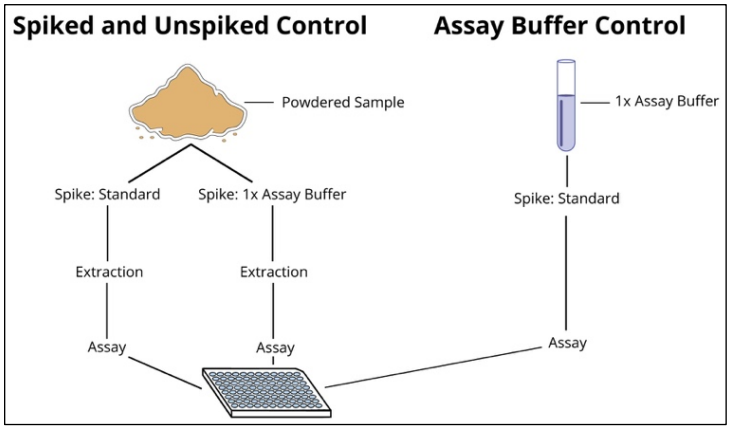

Figure 1. Extraction efficiency workflow diagram for fecal samples.

SAMPLE ANALYTE CONCENTRATION CALCULATION

For fecal samples, concentrations are often reported as the analyte concentration normalized to sample mass (e.g., pg/g of sample). This section outlines the calculation to normalize your sample concentration to sample mass using the formula below and provides an example scenario. Concentration units are assay dependent.

$$\textbf{Normalized Analyte Concentration}\left(\frac{\text{pg}}{\text{g}}\right) = \frac{\left(\text{Assay Concentration}\left(\frac{\text{pg}}{\text{mL}}\right) \times \text{Assay Dilution Factor} \times \text{Total Reconstitution Volume (mL)}\right)}{\left(\text{Supernatant Volume (mL)} \times 0.1\frac{\text{g}}{\text{mL}} \times \text{Extraction Efficiency}\right)}$$

Below is a simplified example extraction and the resulting calculations to normalize sample concentration to sample mass. The volumes described below may need to be adjusted for experimental samples.

1. Extract steroids from 0.2 g dried fecal material using 2.0 mL ethanol.
2. Centrifuge sample and transfer 0.8 mL of supernatant to a clean tube.
3. Evaporate the supernatant completely and reconstitute the extract in 100 µL ethanol and 400 µL 1X Assay Buffer (500 µL Total Reconstitution Volume).
4. Add 25 µL of the reconstituted extract from step 3 to 475 µL 1X Assay Buffer (20x dilution factor). Use this sample to run the assay following the assay protocol.
5. The assay reported a concentration of 100 pg/mL for this sample. Following the equation above and using a 95% extraction efficiency, the normalized concentration to sample mass is:

$$\frac{\left(100\left(\frac{\text{pg}}{\text{mL}}\right) \times 20 \text{ Dilution Factor} \times 0.5 \text{ (mL)}\right)}{0.8 \text{ (mL)} \times 0.1\frac{\text{g}}{\text{mL}} \times 0.95} = 13{,}158 \text{ pg analyte/g sample}$$