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# Quantifying Cellular Lipids

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**Protocol status:** In development
**We are still developing and optimizing this protocol**

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## Disclaimer

This protocol is intended for research use only and is not validated for diagnostic or clinical decision-making. Results may depend on cell type, fixation/permeabilization conditions, and plate materials; optimization in the user's system is recommended.

## Abstract

This protocol provides a fast, plate-based workflow to quantify cellular lipids using polarity-resolved Nile Red fluorescence coupled with crystal-violet (CV) biomass normalization. Fixed adherent cells in well plates are stained with Nile Red and read in situ across defined excitation/emission pairs to estimate neutral-to-polar lipid ratios. In a second step, Nile Red is extracted from the same wells with DMSO to generate a standard-curve–based proxy for total lipid mass. CV staining of the fixed monolayer yields an absorbance metric for per-well biomass, enabling normalization of total lipid measures. The method completes in ~3 hours, requires only standard plate readers and common reagents, and includes quality-control guidance (channel grouping, linear-range checks, and troubleshooting). Outputs include (i) Neutral:Polar fluorescence ratio, (ii) total Nile Red–equivalent lipid signal, and (iii) biomass-normalized indices suitable for comparing treatments, doses, or time courses. The protocol is broadly applicable to lipid metabolism studies, phenotypic screening, and pharmacologic perturbation assays, and is readily scalable to alternate plate formats with proportional volume and readout adjustments.

# Quantifying Cellular Lipids

1 **Quantifying Cellular Lipids with Nile Red and Crystal-Violet Normalization (adherent cells, 12/24-well format)**

**Overview:** This protocol combines (i) polarity-resolved *in-situ* Nile Red scanning to estimate neutral:polar lipid ratios, (ii) bulk DMSO extraction to quantify total lipid mass against a Nile Red standard curve, and
(iii) crystal-violet staining to normalize lipid readouts to fixed-cell biomass. Optimized for adherent cells grown in 12-well plates and read on a monochromator-based microplate reader.

**At-a-glance**

- **Species/Material:** Adherent mammalian cells
- **Plate format:** 12-well (clear-bottom, tissue-culture treated)
- **Reader optics:** Bottom read; monochromator recommended
- **Total hands-on time:** ~2.5–3 h (excluding cell culture

2 **Safety**

- Handle PFA and crystal violet in a fume hood; wear gloves, coat, and eye protection.
- DMSO facilitates skin absorption—avoid contact.
- Dispose of PFA, DMSO, dyes, and alcohol per institutional chemical-waste rules.

3 **Materials and Reagents**

- 4% paraformaldehyde (PFA) in PBS (fixative)
- PBS, pH 7.4 (wash/stain buffer)
- Triton X-100, 10% (v/v) stock (for optional permeabilization; use 0.1% working)
- Nile Red (NR), 1 mg/mL in anhydrous DMSO (–20 °C); **working:** 0.5 µg/mL in PBS + 0.1% DMSO
- DMSO (anhydrous; for extraction)
- Crystal violet (CV), 0.1% (w/v) in 20% (v/v) methanol (MeOH)
- CV elution solvent: 95% ethanol (or 10% EtOH + 1% acetic acid)
- Nile Red standards: 0–2 µM NR in DMSO (prepare fresh)
- Deionized water

4 **Consumables & Equipment**

- 12/24-wellclear-bottom plates; black 96-well plates (fluorescence); clear 96-well plates (absorbance)
- Microplate reader with variable excitation/emission (bottom optics; area scan capable)
- Plate shaker / rocker, pipettes, timer, foil to protect from light

5 **Before You Start**

- Culture cells to the desired endpoint in 12/24-well plates (record confluency).

- Warm PBS and PFA to room temperature (RT).
- Prepare fresh NR working solution (0.5 µg/mL in PBS + 0.1% DMSO). Protect from light.
- Program the reader with the channels listed under **Reader Settings**.

6 **Reader Settings (polarity-resolved *in-situ* scan)** For each excitation (Ex), collect three emission (Em) bands: **Em#1 Neutral**, **Em#2 Mixed**, **Em#3 Polar/Total**. Suggested sets:

| | | | | |
|---|---|---|---|---|
| | 485 | 530 ± 5 | 575 ± 5 | 625 ± 5 |
| | 515 | 560 ± 5 | 600 ± 5 | 640 ± 5 |
| | 529 | 575 ± 5 | 610 ± 5 | 645 ± 5 |
| | 554 | 585 ± 5 | 625 ± 5 | 660 ± 5 |
| | 561 | 590 ± 5 | 630 ± 5 | 665 ± 5 |
| | 573 | 605 ± 5 | 640 ± 5 | 675 ± 5 |
| | 594 | 625 ± 5 | 660 ± 5 | 700 ± 5 |

**Area scan (bottom optics):** 25-point grid; integration 100–300 ms; gain/PMT Auto or fixed (record value); temperature ~25 °C. If your instrument allows multiple Ex per scan, include 485 nm (strong neutral-lipid signal) and/or 529 nm as secondary excitations.

## Procedure

7 **A. Fixation (RT; ~40 min)**

1. Aspirate medium; rinse each well **2× with PBS**.
2. Add **500 µL 4% PFA** per well; incubate **30 min** at RT.
3. Discard fixative; rinse **3×** with PBS or water (5 min each).
4. *(Optional)* Permeabilize with **0.1% Triton X-100** in PBS/water for **2 min**; rinse once with PBS.

**Note:** Keep wells covered with liquid at every stage until the *in-situ* scan is complete to avoid drying artifacts.

8 **B. Nile Red Staining — *In-situ* class-specific scan (dark; ~20 min + read time)**

1. Prepare **NR 0.5 µg/mL** in PBS/water + **0.1% DMSO**.
2. Add **400 µL** per well; incubate **10–15 min**, protected from light.
3. Without washing, top up with **≥ 200 µL PBS/water** per well.
4. Read on the plate reader using the **Reader Settings** above; export mean RFU per emission channel per well.

5. **ComputeNeutral:Polar ratio** per well, e.g., **$RFU_{560}$ / $RFU_{640}$** (using the 515 → 560 nm and 515 → 640 nm channels), or a consistent pair across Ex sets.

9 **C. DMSO Extraction — total lipid mass (37 °C; ~25–30 min)**

1. Remove buffer; add **500 µL neat DMSO** to each fixed, stained well.
2. Incubate **15 min at 37 °C**.
3. Transfer **2 × 200 µL** supernatant from each well to a **black 96-well plate**.
4. Measure fluorescence at **Ex 535 nm / Em 630 ± 10 nm**.
5. Run **Nile Red standards (0–2 µM)** in DMSO in parallel; keep sample RFU in the **linear range (< 1 µM)** by dilution if needed.
6. Convert RFU → **µM NR equivalent** via the standard curve; interpret as **total cellular lipid mass proxy**.

10 **D. Crystal-Violet (CV) biomass normalization (RT; ~45 min)**

1. Return to the original 12-well plate; rinse **1× PBS**.
2. Add **500 µL 0.1% CV**; incubate **20 min** at RT.
3. Rinse gently under a slow PBS stream until runoff is colorless; invert on paper towel to remove excess; **air-dry 15 min**.
4. Add **500 µL 95% EtOH** (or 10% EtOH + 1% acetic acid); shake **10 min**. Transfer **200 µL** eluate to a **clear 96-well plate**; read **Abs 590 nm** (optional reference 620 nm).

## Data Analysis

11 **Channel grouping (for QC and robustness)**

- **Neutral**
  **(N):** 485/530; 515/560; 529/575
- **Mixed**
  **(M):** 515/600; 529/610; 554/625 *(QC/transitional)*
- **Polar/Total**
  **(P):** 485/625; 515/640; 529/645; 554/660; 561/665; 573/675; 594/700. Use a predefined pair (e.g., 515/560 vs. 515/640) across plates/experiments for consistency.

12 **Calculations**

1. **Neutral:Polar ratio (per well)**

$R_{N:P} = RFU_{\text{Neutral channel}}/RFU_{\text{Polar channel}}$

(e.g., **$RFU_{560}$ / $RFU_{640}$**).

**2. Total lipid (NR-equivalent, per well)**

Interpolate sample RFU (Ex535/Em630±10) against the **0–2 µM NR** standard curve; report in **µM NR-eq** (dilution-adjusted).

**3. Biomass-normalized metrics**

- **Total lipid per biomass:** $NR\text{-}eq_{well}/Abs_{590}$
- **Neutral:Polar per biomass (optional):** $R_{N:P}/Abs_{590}$

13 **Quality Control**

- The **Mixed (M)** band should sit between N and P; large drift suggests staining or gain issues.
- Replicate CV **Abs590** variance >10–15% suggests inconsistent rinsing or fixation.

## Timing

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- Fixation: 40 min
- NR stain + *in-situ* read: 20–35 min (depends on scan time)
- DMSO extraction + read: 25–30 min
- Crystal-violet normalization: 45 min

**Total:** ~2.5–3 h (excluding culture)

## Plate Layout Recommendations

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- Include **≥3 blank wells** (no cells) carried through staining/extraction for background subtraction.
- Include **technical triplicates** per condition.
- For the standard curve, reserve **one 96-well plate column** for **0–2 µM NR** (in DMSO).

## Troubleshooting

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| | A | B | C |
|---|---|---|---|
| | Very low *in-situ* signal | Under-staining, photobleaching, high PMT threshold | Protect from light; verify NR 0.5 µg/mL; increase integration time within linearity |
| | High background in P band | Incomplete rinses; plate autofluorescence | Ensure PBS top-up before reading; use low-autofluorescence plates |

| | A | B | C |
|---|---|---|---|
| | Non-linear NR standard curve | Dye aggregation at high µM range | Fit only ≤1 µM range; prepare standards fresh; mix well |
| | CV Abs590 highly variable | Over- or under-rinsing; uneven drying | Standardize rinse volume/time and drying interval; shake elution consistently |
| | DMSO extract weak | Short incubation or low temp | Ensure full 15 min at 37 °C; verify volume transfers |

## Notes & Adaptations

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- Works with other plate formats; scale volumes proportionally.
- If top optics are required, verify signal-to-background and adjust integration.
- Permeabilization is optional; over-permeabilization may elevate P-band signal.