Soft Agar Colony Formation Assay

Anchorage-independent growth assay for transformation and tumorigenicity

Overview

The soft agar colony formation assay is a gold standard method for measuring anchorage-independent growth, a hallmark of cellular transformation and tumorigenicity. Normal cells require attachment to a solid substrate for growth, while transformed cells can proliferate in semi-solid medium.

Principle:

Cells are suspended in low-concentration agar (0.3-0.6%) layered over a higher-concentration base agar (0.5-1.0%). Transformed cells form colonies in suspension, while normal cells cannot proliferate without attachment. Colony formation is quantified after 2-4 weeks.

Applications

- Assessing tumorigenic potential
- Testing tumor suppressor function
- Oncogene transformation studies
- Drug screening for cancer cells
- Evaluating metastatic potential
- Quality control for cell lines

Advantages

- Highly predictive of in vivo tumorigenicity
- Quantitative and reproducible
- Can assess thousands of cells per well
- Does not require animal models
- Suitable for high-throughput screening

Expected Timeline:

- **Day 0:** Prepare agar layers and seed cells
- **Day 1-3:** Monitor for contamination and agar integrity
- **Week 2-3:** Colonies become visible (>50 µm)
- **Week 3-4:** Count and analyze colonies
- **Total duration:** 21-28 days

Important Considerations

- Agar must remain molten (42°C) but not so hot it kills cells
- Work quickly to prevent agar from solidifying prematurely
- Maintain sterile technique throughout - assay lasts 3-4 weeks
- Use low-passage cells for reproducible results
- Include positive and negative controls

Required Materials

Reagents

- **Noble Agar** or agarose (low melting point)
- Cell culture medium (DMEM, RPMI, etc.)
- Fetal bovine serum (FBS)
- Penicillin/Streptomycin
- L-Glutamine or GlutaMAX
- PBS (phosphate buffered saline)
- Trypsin-EDTA or cell dissociation reagent
- Nitroblue tetrazolium (NBT) or MTT (for staining)
- Sterile water or PBS for agar preparation

Equipment & Supplies

- 6-well cell culture plates
- Biological safety cabinet
- 37°C CO_2 incubator with humidity control
- Water bath (42°C)
- Microwave or autoclave
- Hemocytometer or cell counter
- Sterile pipettes and tips

- Inverted microscope
- 50 mL conical tubes
- Plate reader or colony counter (optional)
- Image analysis software (ImageJ, etc.)

Cell Requirements

- Test cells: Transformed or tumor cell lines
- Positive control: Known transformed cell line (HeLa, HT-1080)
- Negative control: Normal, non-transformed cells (NIH 3T3, MCF-10A)
- Cells should be in log-phase growth
- Viability >95% required
- Use cells at consistent passage number

Volumes per 6-well Plate

Base Layer (1% agar): 2 mL per well

Top Layer (0.6% agar + cells): 1.5 mL per well

Feed medium: 0.5-1 mL per well (added weekly)

Note: Scale proportionally for other plate formats

Agar Preparation (Day Before or Day Of)

> **Important: Prepare agar stocks in advance. They can be stored and remelted as needed.**

1. Prepare 2% Noble Agar Stock

Mark Done

- Weigh 2 g Noble Agar powder
- Add to 100 mL sterile water or PBS in autoclavable bottle
- Autoclave at 121°C for 15-20 minutes OR
- Microwave in 30-second bursts until fully dissolved (mix between bursts)
- Store at room temperature (will solidify)
- Can be stored for several months and remelted

2. Prepare 2× Complete Medium

Mark Done

- Prepare complete medium with double-strength supplements

- Example for DMEM: 2× DMEM powder + 20% FBS + 2× Pen/Strep + 2× L-glutamine
- Or: Mix 1:1 regular medium concentrate with water
- Sterile filter (0.22 µm)
- Store at 4°C

3. Prepare Base Agar (1% - Day of Plating)

Mark Done

- Melt 2% agar stock in microwave or boiling water bath
- Pre-warm 2× complete medium to 42°C
- In BSC, mix equal volumes: 2% agar + 2× medium
- Example: 10 mL agar + 10 mL 2× medium = 20 mL of 1% base agar
- Mix thoroughly but gently (avoid bubbles)
- Keep at 42°C in water bath until ready to plate

4. Prepare Top Agar (0.6% - Day of Plating)

Mark Done

- Melt 2% agar stock
- Pre-warm 2× complete medium to 42°C
- Mix 0.6% agar: 3 parts 2× medium + 1 part 2% agar
- Example: 15 mL 2× medium + 5 mL 2% agar = 20 mL of 0.6% agar
- Alternative: 6 mL agar + 14 mL 2× medium = 20 mL of 0.6%
- Keep at 42°C until ready to mix with cells

Quick Calculation Reference

For 6-well plate (6 wells):

- Base layer (1%): Need 12-14 mL total (2 mL × 6 wells + extra)
- Top layer (0.6%): Need 10-12 mL total (1.5 mL × 6 wells + extra)

Soft Agar Assay Protocol

> **Day 0: Plating (work quickly once agar is melted - it solidifies rapidly below 40°C)**

1. Prepare Cells

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- Harvest cells in log-phase growth by trypsinization
- Neutralize trypsin with complete medium
- Count cells and assess viability (should be >95%)
- Prepare single-cell suspension (no clumps)
- Typical concentration: **5,000-10,000 cells per well** (optimize for your cell line)

2. Plate Base Agar Layer

Mark Done

- Bring 1% base agar to BSC (keep at 42°C)
- Pipette **2 mL per well** into 6-well plate
- Swirl plate gently to ensure even coverage
- Allow to solidify at room temperature (10-15 minutes)
- Do NOT refrigerate - will crack
- Base layer should be firm before adding top layer

3. Mix Cells with Top Agar

Mark Done

- Dilute cells in pre-warmed (37°C) complete medium
- Calculate volume needed: cells should be in same volume as top agar
- Example: For 10 mL top agar, prepare cells in 10 mL medium
- In sterile tube, mix cells + 0.6% top agar (42°C) at 1:1 ratio
- Final: cells in 0.3% agar in complete medium
- Mix gently but thoroughly by pipetting
- **Work quickly - agar solidifies rapidly!**

4. Plate Top Layer with Cells

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- Immediately pipette **1.5 mL cell/agar mixture per well**
- Dispense gently onto center of solidified base layer
- Tilt plate gently to spread evenly (don't swirl vigorously)
- Work well-by-well to prevent agar solidification in tube
- Allow top layer to solidify at room temperature (10-15 minutes)

5. Add Feed Layer (Optional but Recommended)

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- After top layer solidifies, add 0.5-1 mL complete medium on top
- This prevents agar from drying out
- Provides additional nutrients
- Feed weekly by adding fresh medium (don't remove old medium)

6. Incubation

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- Place plates in 37°C humidified CO_2 incubator
- Incubate for **14-28 days** (typically 21 days)
- Check plates every 2-3 days for contamination
- Monitor agar for drying (add medium if needed)
- Colonies typically visible by day 10-14 under microscope

7. Weekly Feeding

Mark Done

- Once per week, add 0.5 mL fresh complete medium per well

- Do NOT remove existing medium - just add fresh on top
- This provides nutrients and prevents drying
- Be gentle - don't disturb agar layers

Colony Quantification and Analysis

Timing: Begin analysis after 14-21 days when colonies are clearly visible (>50 µm diameter)

Method 1: Direct Microscopic Counting (No Staining)

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- Use inverted microscope with 4× or 10× objective
- Count colonies >50 µm diameter (>50 cells)
- Scan entire well systematically in a grid pattern
- Count each well in triplicate (technical replicates)
- Take representative images for documentation

Method 2: Nitroblue Tetrazolium (NBT) Staining

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- Prepare NBT solution: 1 mg/mL NBT in PBS
- Add 0.5 mL NBT solution per well
- Incubate at 37°C for 2-4 hours
- Living colonies stain dark blue/purple
- Remove NBT and image plates on white background
- Count stained colonies visually or with image analysis software

Method 3: MTT Staining (Colorimetric)

Mark Done

- Add MTT reagent (0.5 mg/mL final) to wells
- Incubate 2-4 hours at 37°C
- Colonies stain purple
- Can extract formazan with DMSO for quantitative absorbance reading

Image Analysis Quantification

Mark Done

- Photograph entire well with consistent lighting
- Use ImageJ/Fiji or CellProfiler for automated counting
- Set size threshold (typically >50 µm diameter)
- Automated counting reduces user bias and speeds analysis

Data Analysis and Reporting

- Report as: **Number of colonies per well** or **Colony formation efficiency (%)**
- Colony formation efficiency = (# colonies / # cells plated) × 100
- Use at least 3 biological replicates with 2-3 technical replicates each
- Include representative images at same magnification
- Statistical analysis: t-test, ANOVA, or non-parametric tests
- Consider both colony number AND colony size for comprehensive analysis

Troubleshooting

Problem	Possible Cause	Solution
No or very few colonies	Cells not transformed Too few cells plated Low viability Agar concentration too high	Verify cell transformation status with positive control Increase cell number (try 5,000-20,000/well) Check viability before plating Reduce top agar to 0.3-0.35%
Colonies too small	Insufficient incubation time Nutrient depletion	Extend incubation to 28 days Feed more frequently (2× per week) Increase feed volume
Agar dried out	Insufficient feed medium Low humidity in incubator	Add more medium on top Place water pan in incubator Use plates on bottom shelves
Agar cracked	Refrigeration Agar too thick	Never refrigerate agar plates Reduce agar layer thickness Ensure complete solidification before incubating
Cells settled to bottom	Agar too hot when mixed Agar solidified too slowly	Cool agar to 40-42°C before mixing cells Plate immediately after mixing Work in cooler room if needed
Uneven colony distribution	Cell clumping Inadequate mixing	Ensure single-cell suspension Mix cells thoroughly but gently Pipette while dispensing
Contamination	Breach of sterility Long incubation	Strict aseptic technique Add pen/strep to media

Problem	Possible Cause	Solution
	period	Seal plate edges with parafilm Use antibiotic/antimycotic
Monolayer growth on plate bottom	Cells attached to plastic Base layer too thin	Increase base layer thickness Ensure base layer fully covers well Check for agar gaps

Optimization Tips

- Test range of cell numbers: 1,000-20,000 per well
- Optimize agar concentration (0.3-0.6% top layer)
- Some cells form colonies faster (2 weeks vs 4 weeks)
- Use low-passage cells for consistency
- Include positive control (known transformed line)
- Include negative control (normal cells)

Best Practices

- Always prepare fresh agar/media mix
- Work quickly once agar is molten
- Maintain consistent temperature (42°C)
- Don't disturb plates during incubation
- Count colonies at consistent time point
- Use blinded counting when possible