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Expansify™ Cell Culture Methods- HEK293T Expansion

Based on internally validated culture conditions and representative experimental data generated using Expansify™ plates

Purpose

This culture guide provides representative conditions for the expansion and maintenance of HEK293T cells in Expansify™ 24- and 1-Well Plates. The protocol is based on internally generated experimental data and includes representative morphology, seeding conditions, media exchange recommendations, and expected growth characteristics observed under XDemics validated laboratory conditions. Further optimization may be required based on the intended application and desired outcome.

Materials

Prior to use, review the Expansify™ Quick Start Guide and instructional video series in the resources section of the XDemics website.

- Expansify™ 24-Well Plate (Cat. No. 24W-R-01-01) or 1-Well Plate (Cat. No. 01W-R-01-01)
- Cell line: HEK293T (ATCC® CRL-3216™).
- Single-use glass or polystyrene Pasteur pipettes are required for fluid handling (e.g., CELLTREAT® Polystyrene Pasteur Pipette, Cat. No. 229280).
- Culture medium: DMEM High Glucose (Gibco, Cat. No. 11965092) supplemented with 10% Fisherbrand™ Research Grade Fetal Bovine Serum, Canadian Sourced (Cat. No. FB12999102).
- Phosphate-buffered saline (PBS), Ca^{2+}/Mg^{2+}-free (Gibco, Cat. No. 10-010-023)
- EDTA (0.5 M), pH 8.0, RNase-free (Invitrogen™, Cat. No. AM9260G)
- Dissociation reagent required for this cell type: StemPro™ Accutase™ Cell Dissociation Reagent (Gibco, Cat. No. A1110501)
- Extracellular matrix (ECM) coating reagent: Vitronectin (VTN-N) Recombinant Human Protein, truncated (Gibco, Cat. No. A400458)
- Wide-bore P1000 pipette tip (recommended)
  NOTE: Equivalent products from other manufacturers may be substituted for the culture medium, FBS, Ca^{2+}/Mg^{2+}-free PBS, EDTA, Accutase™ cell dissociation reagent, vitronectin coating reagent, and wide-bore P1000 pipette tips listed above. All other materials should be used as specified, including the listed manufacturer and catalog number.

Experimental Procedure

Day -1: ECM Coating

1. Prepare ECM-coated Expansify™ plates according to **Expansify™ Technical Note - Extracellular Matrix (ECM) Coating of Expansify™ Plates**.

Day 0: Culture Initiation

1. Aspirate the ECM coating solution according to the Expansify™ plate format:
    a. 24-Well Plate: Aspirate the coating solution using the 0.25 mL SureShelves™.
    b. 1-Well Plate: Aspirate the coating solution through SureShelves™ A. The plate may be tilted slightly toward the SureShelves™ to facilitate removal of the remaining coating solution.
2. Immediately add complete culture medium:
    a. **24-Well Plate:** 1 mL per well.
    b. **1-Well Plate:** 35 mL per plate.
3. Inspect the bottom of the niches for trapped air bubbles. If air bubbles are visible, centrifuge the plate at 200 × g for 1 minute using an appropriate Expansify™ plate or deep-well plate as a balance. Confirm that the niches are fully wetted before proceeding.
    NOTE: Following ECM coating, the culture surface is typically already wetted, and centrifugation may not be necessary. However, occasional air bubbles may remain or become trapped within the niches. These bubbles can prevent complete filling of the niche and reduce the surface area available for cell attachment and growth.
4. Seed HEK293T cells at 2.5 × 10^4 cells/cm^2 into the desired wells of the Expansify™ 24-Well Plate or 1-Well Plate.
5. Adjust the final culture volume to:
    a. **24-Well Plate:** 4.5 mL per well.
    b. **1-Well Plate:** 100 mL per plate.
6. Following seeding, gently homogenize the cell suspension to promote uniform cell distribution and settling throughout the culture niches.
7. Monitor and image cultures as needed throughout the culture period (e.g., Days 1–7).

Days 5, 6 and 7: Medium Exchange

1. Perform complete medium exchanges on Days 5, 6, and 7 (e.g., red dotted arrow lines in growth curve below):
    a. **24-Well Plate:** Keep the plate level on the work surface and aspirate the spent medium using the 0.25 mL SureShelves™. Add 4.5 mL of fresh complete medium using the 0.25 mL or 1 mL SureShelves™.
    b. **1-Well Plate:** Keep the plate level and remove spent medium through SureShelves™ A. Once most of the medium has been removed, the plate may be tilted approximately 5° toward the shelf to facilitate removal of the remaining ~20 mL. Add 100 mL of fresh complete medium through SureShelves™ A.

TIP: For the 1-Well Plate, use the same designated locations for medium addition and aspiration during each exchange to minimize disruption to the culture. For example, consistently add medium from one side and aspirate from the opposite SureShelves™.

Day 4–8: Cell Harvest

1. Harvest cells according to the Expansify™ plate format being used:
    a. **24-Well Plate (Days 4, 5, 6, 7 and 8):**
        i. **Wash the culture:** Keep the plate level on the work surface. Aspirate the spent medium using the 0.25 mL SureShelves™. Add an excess volume of wash buffer, > 2 mL per well, such as 1× PBS (Ca^{2+}/Mg^{2+}-free) supplemented with EDTA to a final concentration of 5 mM, using the 0.25 mL or 1 mL SureShelves™, then aspirate the wash buffer using the 0.25 mL SureShelves™.
        ii. **Initiate cell dissociation:** Add StemPro™ Accutase™ Cell Dissociation Reagent to each well to be harvested according to the anticipated cell number, estimated based on the expected doubling time and duration of culture:
            1. **≤ 2×10^6 cells/well:** 1 mL
            2. **> 2×10^6 to < 1×10^7 cells/well:** 2–2.5 mL
            3. **≥ 1×10^7 cells/well:** 2.5–3 mL

            Gently mix up and down 2–3 times using a P1000 wide-bore pipette tip to distribute the enzyme throughout the well, then incubate at 37°C and 5% CO_2 for 7–10 minutes.
        iii. **Assess dissociation:** Remove the plate from the incubator and gently mix the culture using a P1000 wide-bore pipette tip. Examine the cells under a microscope to assess dissociation. If a uniform single-cell suspension has not been achieved, return the plate to the incubator for an additional 7–10 minutes, then repeat the gentle mixing step. Avoid excessive mixing or mechanically forcing dissociation, as this may increase shear stress and reduce cell viability.
            NOTE: The recommended StemPro™ Accutase™ Cell Dissociation Reagent volumes and incubation conditions are based on XDemics' internal validation. Under these conditions, a uniform single-cell suspension should typically be achieved within a cumulative incubation time of approximately 30 minutes at the highest cell densities evaluated in Expansify™, with shorter incubation times generally sufficient at lower cell densities. If additional dissociation is required, repeated incubation and gentle mixing steps may be performed as needed. Total incubation time at 37°C should not exceed 45 minutes, as Accutase™ progressively self-inactivates and loses enzymatic activity over this period.
        iv. **Collect the cells:** Collect the resulting cell suspension for downstream processing, analysis, or further expansion.

NOTE: StemPro™ Accutase™ Cell Dissociation Reagent does not require neutralization. Complete culture medium may be added if desired. Record the final harvest volume prior to downstream processing.

b. **1-Well Plate (Day 7):**

i. **Wash the culture:** Keep the plate level on the work surface. Aspirate the spent medium through the right-side SureShelves™ A. Once most of the medium has been removed, the plate may be tilted approximately 5° toward the aspiration side to facilitate removal of the remaining liquid. Return the plate to a level position, then add an excess volume of wash buffer, >40 mL, such as 1× PBS (Ca^{2+}/Mg^{2+}-free) supplemented with EDTA to a final concentration of 5 mM, through the left-side SureShelves™ A. Aspirate the wash buffer through the right-side SureShelves™ A. Once most of the wash buffer has been removed, tilt the plate approximately 5° toward the aspiration side to remove as much residual wash buffer as possible and minimize dilution of the dissociation reagent used in the subsequent step.

TIP: For the 1-Well Plate, use the same designated locations for medium addition and aspiration during each exchange to minimize disruption to the culture. For example, consistently add medium from one side and aspirate from the opposite SureShelves™.

ii. **Initiate cell dissociation:** Add StemPro™ Accutase™ Cell Dissociation Reagent to the plate to be harvested according to the anticipated cell number, estimated based on the expected doubling time and duration of culture:

1. **≤ 7×10^7 cells:** 35 mL
2. **> 7×10^7 to < 3.5×10^8 cells:** 35–70 mL
3. **≥ 3.5×10^8 cells:** 70–100 mL

Gently mix using a 25 mL or 50 mL serological pipet to distribute the enzyme throughout the culture, then incubate at 37°C and 5% CO_2 for 7–8 minutes.

iii. **Assess dissociation:** Remove the plate from the incubator and gently mix the culture using a 25 mL or 50 mL serological pipet. Examine the cells under a microscope to assess dissociation. If a uniform single-cell suspension has not been achieved, return the plate to the incubator for an additional 7–10 minutes, then repeat the gentle mixing step. Avoid excessive mixing or mechanically forcing dissociation, as this may increase shear stress and reduce cell viability.

NOTE: The recommended StemPro™ Accutase™ Cell Dissociation Reagent volumes and incubation conditions are based on XDemics' internal validation. Under these conditions, a uniform single-cell suspension should typically be achieved within a cumulative incubation time of approximately 30 minutes. If additional dissociation is required, repeated incubation and gentle mixing steps may be performed as

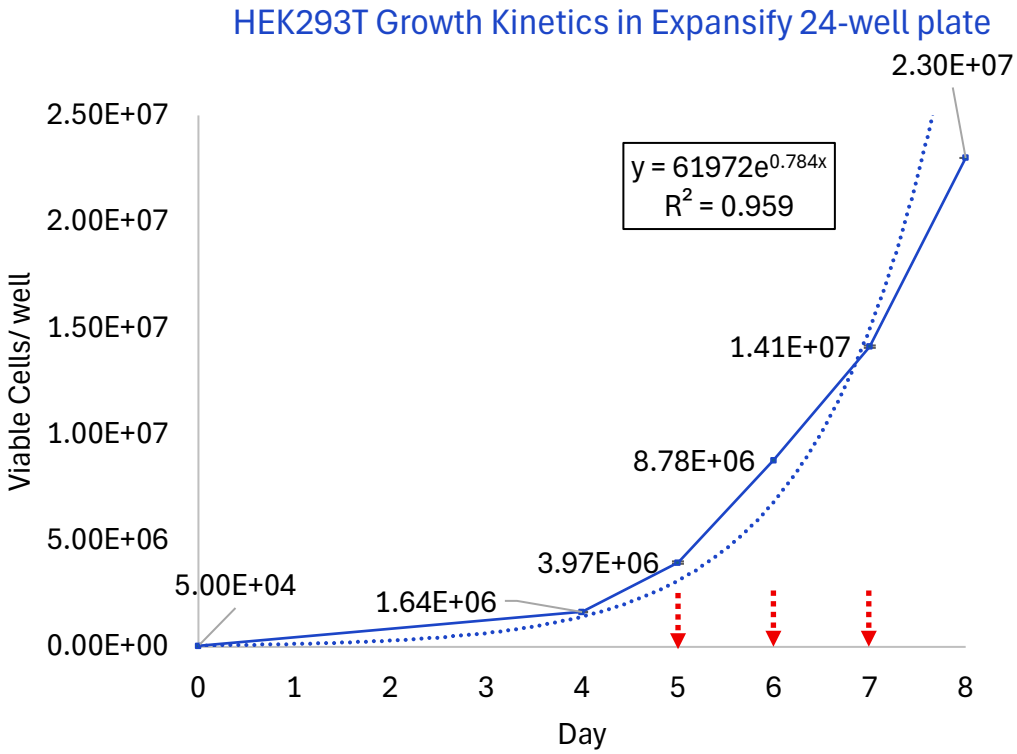
needed. Total incubation time at 37°C should not exceed 45 minutes, as Accutase™ progressively self-inactivates and loses enzymatic activity over this period.

iv. **Collect the cells:** Collect the resulting cell suspension for downstream processing, analysis, or further expansion.
<u>NOTE:</u> StemPro™ Accutase™ Cell Dissociation Reagent does not require neutralization. Complete culture medium may be added if desired. Record the final harvest volume prior to downstream processing.

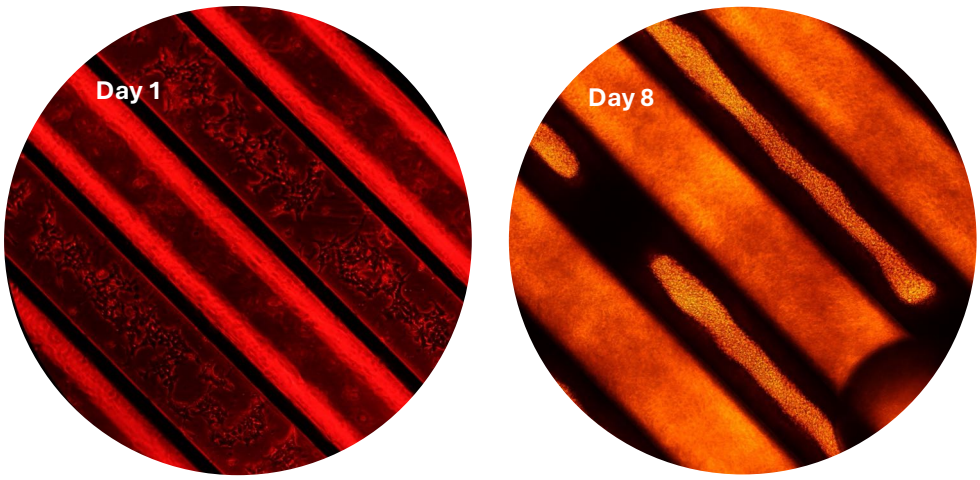
Performance Summary

24-Well Plate: Cells reached >2 × 10^7 cells per well (see growth curve below), with a mean doubling time of 21.22 h over 8 days. This corresponded to an approximately 460-fold expansion. Data represent two independently harvested wells per time point (n = 2), with separate wells destructively harvested at each time point. Cell viability was >90% at all time points, as assessed by AOPI staining using the Cellaca MX cell counter with the built-in "Cell Lines" assay.

1-Well Plate: In a representative Expansify™ 1-Well Plate (n = 1), 1.75 × 10^6 cells expanded into 5.36 × 10^8 cells by Day 7, corresponding to an approximately 306-fold expansion and a terminal doubling time of 21 h. The culture was maintained without intermediate harvests and destructively harvested on Day 7. Cell viability was >90% at the time of harvest, as assessed by AOPI staining using the Cellaca MX cell counter with the built-in "Cell Lines" assay.

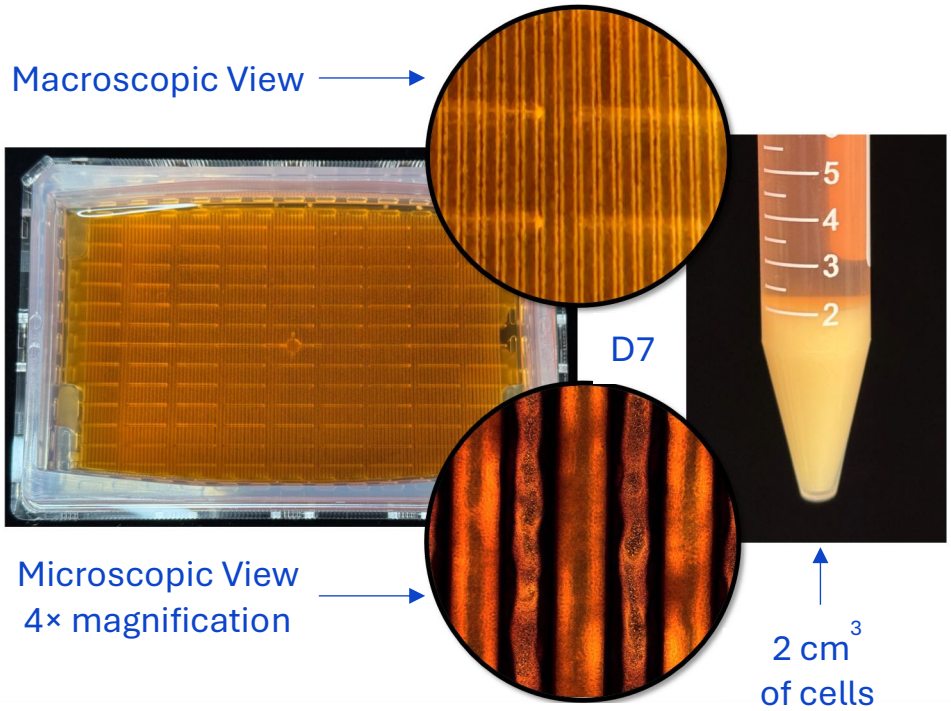


HEK293T Expansion in Expansify™ 24-Well Plate



Representative images of HEK293T cells exhibiting strong adherent morphology 1 day post-seeding. Following initial localization within the culture niches, HEK293T cells underwent rapid expansion and progressively populated the oxygenating surfaces over the 8-day culture period. Images were acquired using an ECHO Revolve microscope at 4× magnification.

HEK293T Expansion in Expansify™ 1-Well Plate



Representative images of HEK293T cultures on Day 7 post-seeding in the Expansify™ 1-Well Plate. The macroscopic view shows extensive cell coverage, while the 4× microscopic view highlights dense growth within the culture niches. Day 7 harvest yielded approximately 2 cm^3 of packed cells. Microscopic images were acquired using an ECHO Revolve microscope at 4× magnification.