HEKxpress Application Note

for Product Codes:

10-02S200	HEKxpress ready-to-use
5-03Z01	HEKxpress Feed with stable glutamine

1. Product description

Components and specifications
With stable L-glutamine
Chemically defined
Free of animal-derived components
Free of hydrolysates, peptides or proteins.

Storage
Store protected from light at 2-8°C. Do not freeze.

Field of use
HEKxpress is exclusively used for *in vitro* research and
for further manufacturing processes **only**.

2. Application

The HEKxpress medium system contains a basal but complete medium for batch cultivation and a feed medium for long-term fed-batch cultivations of HEK239-derived cell lines. Both media formulations are chemically defined, animal component-free, protein-free and hydrolysate-free. The HEKxpress system supports the cell growth and production of e.g. viral vectors or vaccines, recombinant proteins and antibodies in suspension culture. It can be used in research or in manufacturing applications. The HEKxpress batch medium was designed to be compatible with various protocols in transient transfection e.g. by using Polyethylenimin (PEI).

3. Protocols

3.1 Preparations

All procedures should be carried out using sterile techniques in a biosafety cabinet.

The HEKxpress media system contains L-alanyl-L-glutamine for improved stability of the L-glutamine source as well as for convenience. No additional supplementation is necessary.

3.2 General culture conditions

Cultures should be maintained at 37°C with 5% CO_2 and >80% humidity. For shaken cultures, the shaker throw/speed is 50mm/125rpm (Erlenmeyer flask) and 20-50mm/180-220rpm (filter tubes), respectively. The ideal parameters depend on cell line, vessel and/or device and could differ.

3.3 Cultivation, production and cell maintenance

3.3.1 Thawing of frozen aliquots

1. Quickly thaw a vial of frozen cells in a 37°C water bath.

2. Transfer the cells aseptically to a centrifuge tube containing 10mL of HEKxpress batch medium at or below room temperature.

3. Centrifuge cell suspension at 200-300·g for 5 minutes.

4. Aspirate supernatant completely and discard.

5. Resuspend the cells in appropriate volume of HEKxpress batch medium to reach $5x10^5$ cells·mL^{-1} or the desired cell density appropriate for the HEK cell line ($2.5\text{-}7.5x10^5$ cells·mL^{-1}).

6. Transfer cell suspension into an agitated cultivation system of polycarbonate or polypropylene (e.g. 125mL Erlenmeyer flasks or 50mL filter tubes) and place into incubator at 37°C and 5% CO_2.

7. Take an aliquot before for initial for assessment of cell density as well as viability and determine the parameters again after 3-4 days.

8. Proceed with adaptation (3.3.2) or expansion (3.3.3).

3.3.2 General adaptation process from serum-containing cultures or serum-free culture

1. Directly thaw a cryoconserved aliquot in 10 ml HEKxpress batch medium. **

2. Centrifuge at 200-300·g for 5 minutes.

3. Passage cells or change medium by centrifugation every two to four days depending on cell density (recommended $2.5\text{-}5.0x10^6$ cells·mL^{-1}; below $1.0x10^6$ cells·mL^{-1} needs centrifugation).

4. Continue passaging for approx. 2-4 passages until viabilities stabilize at > 90%.

** We recommend to directly thaw the cells into fresh and chilled (10-25°C) HEKxpress batch medium. After about 4 passages in HEKxpress batch medium, the adaptation is successfully established. Serum-containing cultures may take longer to adapt.

3.3.3 Cell expansion and fed batch

1. Cell expansion is performed in a preferably shaken or rocking cultivation system. Here, the HEKxpress batch medium is preincubated to reach at least 15-18°C, but optimally 36-37°C.***

2. Determine viable cell density in the pre-culture in exponential phase (4-6x10^6 cells·mL^{-1}) and inoculate the equilibrated cultivation system yielding a cell density of about 5x10^5 cells·mL^{-1} (operational range 2.5-7.5x10^5 cells·mL^{-1}).

3. At day three, the cell suspension is supplemented with 5% (v/v) HEKxpress feed medium. Repeat the feeding every day or apply the feed in a continuous manner****

4. Suggested starting parameters for bioreactor cultivations of HEK cells using the HEKxpress media system are pH 7.0-7.3, 40-50% Dissolved Oxygen (DO), and a temperature of 36.5-37.5°C.

*** The use of 2-8°C cold culture medium directly from refrigerator was found to be beneficial sometimes. This procedure eliminates handling variations of the medium in the pre-equilibration phase of the medium. We still recommend the slight preincubation to ensure a shorter lag phase.

**** The HEKxpress feed medium was designed to support the growth of HEK293-derived cell lines to an integral cell density of around 10^8 cells·d·mL^{-1} by using 5% (v/v) based on the initial culture volume. The batch medium should not **be** used for cell cultures above an integral cell density of 2.5x10^7 cells·d·mL^{-1}. because cell viability may drop fast in a cell line-dependent manner. **The addition of the HEKxpress Feed medium is recommended before the culture reaches an integral cell density of 5x10^6 cells·d·mL^{-1}. An increase in daily feed addition might be beneficial for cell lines with a doubling time below 18-20 hours (up to 8-10% (v/v)).** The culture can be prolonged by monitoring L-glutamine as well glucose and adjust to a constant level of 1-2mM and 1-3g/L, respectively.

3.3.4 Freezing of cells

The use of serum is not needed for the generation of long-term cryopreserved aliquots.

1. Cell cultures designated to be frozen are cultured for 2-4 days after passaging to reach exponential phase with viabilities above 95%.

2. Centrifuge the cells at 200-300·g for 5 minutes.

3. Resuspend the cell pellet at 10^7-10^8 cells·mL^{-1} in a freezing medium freshly prepared and consisting of 90% cold HEKxpress batch medium and 10 % dimethyl sulfoxide (DMSO; cell culture grade).

4. Place the vials in a pre-cooled (2-8°C) freezing module and incubate for at least 10 hours at -65°C to -80°C. Alternatively, incubate the aliquots for 40-45 minutes at -25°C to -15°C and transfer them seamlessly to a device at -65°C to -80°C for at least 10 hours.

5. Transfer the aliquots to a system providing constantly -140°C to -196°C for long term storage.

3.3.5 Transient transfection

Briefly below, a recommended transfection protocol is described:

1. Day -2 to -3 – Inoculation:

Inoculate the appropriate volume of HEKxpress with $5x10^5$ cells·mL^{-1} (operational range 2.5-$7.5x10^5$ cells·mL^{-1}) two to three days prior to the transfection. CRUCIAL: *Prior to this step, the HEK293 cells need to be adapted in HEKxpress for at least two passages (Cultivation and adaptation according to chapter 3.3.3, section 1 and 2).*

2. **Day 0** – Transfection:

a. Harvest the HEK293 cells, which reached exponential growth in HEKxpress at the cell density of 2.5-$5x10^6$ cells·mL^{-1}.
b. Depending on your desired transfection culture volume, dispense the necessary cell suspension in a new vessel and centrifuge the cell culture at 200-350·g, five minutes and room temperature.
   CRUCIAL: *Use a fresh vessel and fully aspirate the culture medium. DO NOT use conditioned media or batch cultures, since the containing metabolites and proteins may inhibit the transfection process.*
c. Resuspend the pellet with fresh RPMI1640 medium to establish a cell density of $3x10^7$ cells·mL^{-1}.
   CRUCIAL: *The cell density varies from cell line to cell line, but $3x10^7$ cells·mL^{-1} is the optimised setting for most commercially available HEK293 cells. Any variation may lead to a reduction in performance.*
   NOTE: *To reduce shear stress, 0.1% poloxamer 188 can be added. We do not recommend the addition of HEPES, since it has been shown to slightly inhibit the performance.*
d. Add 0.5µg plasmid DNA/10^6 cells (e.g. 15µg per $3x10^7$ cells and 10mL final volume) to the cell suspension and swirl gently.
   CRUCIAL: *Directly add the DNA to the cell suspension at this concentration. Any variation may lead to a reduction in performance.*
   CRUCIAL: *If you use multiple plasmids, mix all plasmids together prior to the addition to the cell suspension. Individual additions may lead to variations in expression as well as performance.*
   NOTE: *We recommend plasmid vectors including episomal replication, optimised codon sequences as well as strong promotors to guarantee the maximal expression.*
e. Subsequently, add HEKxpress Transfection Reagent (2.25µL/10^6 cells) and swirl gently.
   CRUCIAL: *The Reagent to DNA ratio is optimised. Any variation may lead to a reduction in performance.*
f. The culture is then transferred into an incubator at 37°C and 5% CO_2 and incubated for 45–90 min under constant agitation at 80-220 rpm by an orbital shaker with 20-50 mm amplitude, depending on the vessel.
g. After incubation, the culture is diluted with 9-times the volume of prewarmed HEKxpress adjusting the cell density to $3x10^6$ cells·mL^{-1} (e.g. 9mL HEKxpress to yield 10mL final volume, see table 1).

h. The culture is then transferred back into the incubator at 37°C and 5% CO_2 and incubated for at least 40 hours under constant agitation at 120-220 rpm by an orbital shaker with 20-50 mm amplitude.
CRUCIAL: Any manipulation before 40 hours may cause a reduction in performance.

Table 1: Volumes and amounts for the HEKxpress transfection process.

Vessel	Cell number	RPMI1640	pDNA	HEKxpress Transfection Reagent	HEKxpress Medium
50 mL Bioreactor Tube	3 x10^7	1 mL	15 µg	67.5 µL	9 mL
125 mL Erlenmeyer flask	7.5 x10^7	2.5 mL	37.5 µg	168.75 µL	22.5 mL
1000 mL Erlenmeyer flask	6 x10^8	20 mL	300 µg	1350 µL	180 mL
450/600 mL Bioreactor Tube	1.2 x10^9	40 mL	600 µg	2700 µL	360 mL

3. **Day 2, day 3 and day 4** –Fed batch with boost:

To boost the productivity and longevity of the production process, add the HEKxpress Feed at 5% (v/v) as well as the HEKxpress Boost at 0.25% (v/v). We recommend the daily addition of HEKxpress Feed as well as Boost. For relevant value, see table 2.

CRUCIAL: The boost leads to significantly higher yields. Anyway, do not exceed 1 % (v/v) in a single addition or in a cumulative manner.

Table 2: Parameters for the HEKxpress fed batch production process.

Vessel	Culture Volume	HEKxpress Feed	HEKxpress Boost
50 mL Bioreactor Tube	10 mL	0.5 mL·d^{-1}	25 µL·d^{-1}
125 mL Erlenmeyer flask	25 mL	1.25 mL·d^{-1}	62.5 µL·d^{-1}
1000 mL Erlenmeyer flask	200 mL	10 mL·d^{-1}	500 µL·d^{-1}
450/600 mL Bioreactor Tube	400 mL	20 mL·d^{-1}	1000 µL·d^{-1}

4. **Day 5 to day 8** – Fed batch:

Continue adding the HEKxpress Feed at a daily rate of 5% (v/v). This guarantees the prolonged production of your target protein.

NOTE: Monitor the viability and adjust the process according to your requirements.