Generation of stable mammalian cell lines for the expression of proteins related to COVID-19 through random integration

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Background:

Scientists centred around University of Oxford came together in the current COVID-19 pandemic to contribute to relevant studies collaboratively. Researchers with no prior experience in virology studies, myself included, had volunteered our time to contribute whichever way we can based on our field of expertise. The Biotech team of SGC Oxford, headed by Nicola Burgess-Brown had taken up the role of producing proteins used in serological assays, basic research to understand COVID-19 pathophysiology and hunt for treatment compounds. The initial production of these proteins was based on transient transfection of expression construct DNA into mammalian cells. This approach was laborious and consumed large amounts of plasmid DNA. Therefore, I volunteered to generate stable expression cell lines that will negate the constant need for transfection.

Experimental design:

I aimed to stably integrate the expression constructs into the genome of Expi293F human cells commonly used in large scale protein production. The expression constructs carried neomycin marker for selection in mammalian cells. Toxicity to the cells is not a concern in this case because the expressed proteins are secreted into the medium and do not build up in the cells. The expression constructs are randomly integrated into the genome mainly via non-homologous end-joining (NHEJ). An advantage of this approach is that molecular sub-cloning is not necessary.

In order to maximise the rate of correct integration of the expression constructs, restriction digestion was performed to cut once between the expression cassette and antibiotic resistance cassette (refer to cartoon in Figure 1). Without this step, the construct DNA will be broken at a random position during the integration step, potentially disrupting the expression cassette. I have also tested the effect of actively creating double strand breaks (DSBs) in the genome of the cells on the integration efficiency. More DSBs in the genome might increase the incidence of NHEJ and integration of our constructs. This was achieved by co-transfection of AAVS1 CRISPR/eSpCas9(1.1) construct (refer to cartoon in Figure 1). The eSpCas9(1.1)_No_FLAG_AAVS1_T2 construct was a gift from Yannick Doyon (Addgene plasmid # 79888 ; http://n2t.net/addgene:79888 ; RRID:Addgene_79888)

Detailed protocol:

Adherent growth medium
DMEM high glucose (11995065, Life Technologies)
10% Heat-inactivated FBS (F9665-500ML, Sigma)
10 µg/mL Gentamicin (G1272-10ML, Sigma)

Suspension culture medium
FreeStyle293 expression medium (12338018, Thermo Fisher Scientific)

Cell lysis buffer
Use whole cell lysis buffer without EDTA
50 mM Tris-HCl pH7.4
150 mM NaCl
1% Triton-X100
1 Protease inhibitor cocktail tablet per 10 mL

25 mM NaF
2 mM Sodium Orthovannadate
1:1000 Benzonase (1 µg/mL final concentration)

Cell line to be used
Expi293F (A14527, Thermo Fisher Scientific)

Antibodies used
Anti-Penta-HIS-HRP conjugate kit (34460, Qiagen)

Selection antibiotic
G418 (G8168-10ML, Sigma)

Trypsinisation of adherent Expi293F for reseeding

1. When pipetting, avoid flushing at the adherent cell layer at the bottom of the T75 flask.
2. Remove medium. Rinse cell layer with 3 mL of PBS.
3. Remove PBS and add in 2 mL of TrypLE cell dissociation reagent. Incubate at 37 degree Celsius for 3 minutes.
4. Add 3 mL of growth medium into the flask and pipette up and down 5 times to break down clumps.
5. Centrifuge at 500 x *g* for 3 minutes at room temperature.
6. Discard supernatant and tap to loosen cell pellet.
7. Resuspend cells in growth medium and count. Dilute appropriately to the desired concentration.

Transfection using FugeneHD

1. Pellet Expi293F by centrifugation at 500 x *g* for 3 minutes at room temperature.
2. Resuspend in adherent growth medium and count cells.
3. Adjust to 2e5/mL and aliquot into 10 mL per 50 mL falcon tube (1 for each transfection).
4. Dilute 5 µg linearised plasmid DNA and 0.5 µg CRISPR/Cas9 construct DNA into 500 µL of OptiMEM in an autoclaved 1.5 mL tube.
5. Add 15 µL of FugeneHD reagent (E2311, Promega) without touching the side of the tube.
6. Vortex immediately for 5 seconds.
7. Incubate at room temperature for 20 minutes.
8. Add to 10 mL aliquots of Expi293F cells.
9. Seed into T75 flask.

Cell treatment

1. 48 hours post-transfection, treat cells with 200 µg/mL G418 (G8168-10ML, Sigma).
2. Increase G418 concentration to 800 µg/mL after 3 days and maintain for 9 days with medium change every 3 days.
3. 13 days post-transfection, determine protein expression by Western Blot analysis of the medium.
4. Perform limiting dilution to seed single cells into 96-wells in medium without G418.
5. Identify stable and high expressing clones by Western Blot analysis of the medium 1 and 2 weeks after seeding.
6. Adapt selected clones to suspension growth in FreeStyle293 medium for actual protein purification.

Limiting dilution to obtain single cell clones

1. Trypsinise, resuspend and count cells. Use adherent culture medium for this.
2. Dilute cells step-wise into a) 5 mL of 250,000 cells/mL > b) 1:10 into 5 mL of 25,000 cells/mL > c) 1:10 into 5 mL of 2500 cells/mL > d) 1:10 into 5 mL of 250 cells/mL > e) 1:100 into 30 mL of 2.5 cells/mL.
3. Using sterile reservoir and multichannel pipette, seed 200 µL of the final 2.5 cells/mL suspension into each well of 96-well plate.
4. Keep in plastic bag (without sealing) to minimize evaporation.

Western blot

1. Boil 16 µL of medium in 1X sample buffer (with SDS and 2-ME) and load per well.
2. Transferred gels onto PVDF membrane using Criterion wet transfer system (Biorad) for 1 hour (max current 100 V).
3. Probe membrane using anti-Penta-HIS-HRP conjugate (34460 Qiagen) according to the manufacturer's protocol.
4. Image HRP signal in ImageQuant LAS-4000 using SuperSignal West Pico PLUS Chemiluminescent Substrate (34580, Life Technologies).

Results:

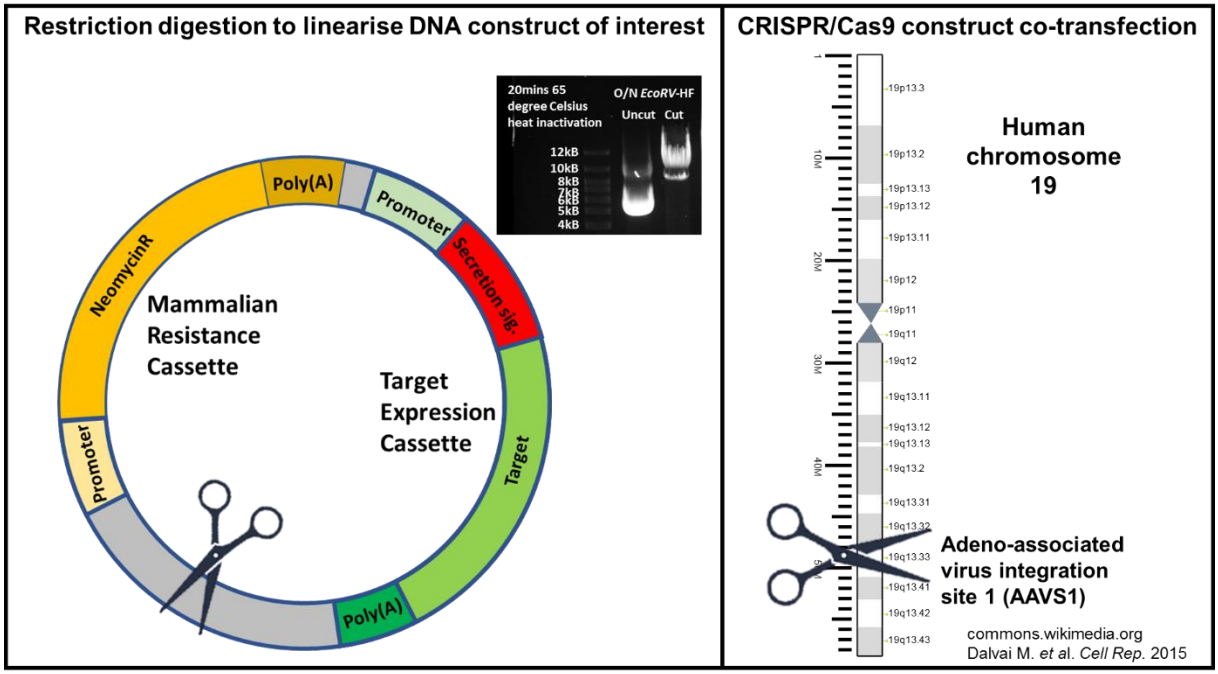

Figure 1. Cartoons of approaches to enhance desired stable integration of protein expression constructs.

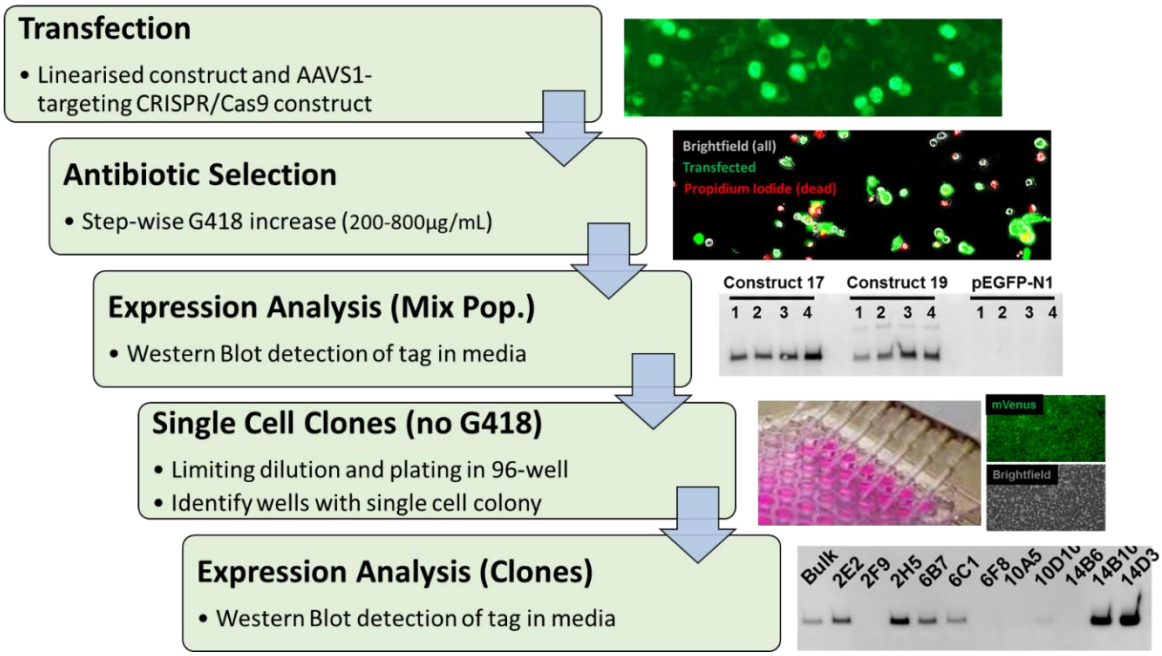

Figure 2. Schematic of the workflow to generate stable cell lines

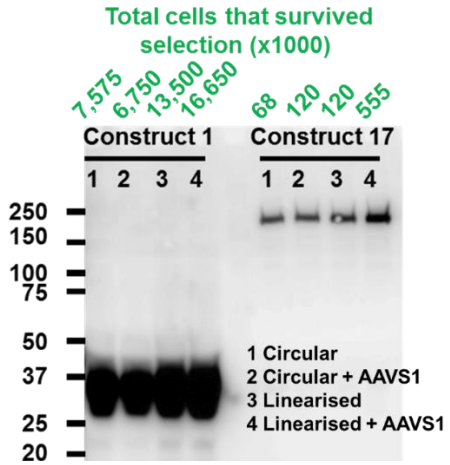

Figure 3. Comparison of the efficiency of correct stable integration under different conditions. Numbers of surviving cells were counted after G418 antibiotic selection and the expression of desired proteins was detected using Western Blot.

Findings:

1. Linearisation of expression construct increased stable integration.
2. Induction of AAVS1 double strand break enhanced stable integration further.
3. The efficiency of stable integration is highly dependent on construct size. E.g. 12.3 kb construct is about 30 times less efficiently integrated than 8.2 kb construct.
4. More than 5-fold increase in purified proteins compared to same volume of transient transfected cells.

In a nut shell:

Stable cells that continuously produce and secrete high level of COVID-19-related proteins have been successfully generated. These cells are suitable for large scale culture and protein purification. These stable cells will help to streamline protein production for serological assays and other COVID-19-related studies.