Please see here for the background to these BACs:

- Stanton, R.J., et al., *Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13 to be a potent inhibitor of replication.* J Clin Invest, 2010.
- Murrell, I. et al. *Impact of Sequence Variation in the UL128 Locus on Production of Human Cytomegalovirus in Fibroblast and Epithelial Cells.* J Virol, 2013
- Murrell I, et al. *Genetic Stability of BAC-Derived Human Cytomegalovirus During Culture In Vitro*. J Virol. 2016.
- Murrell I, et al. *The pentameric complex drives immunologically covert cell -cell transmission of wild-type human cytomegalovirus*. PNAS 2017.

Available BACs

These are the BACs we have available:

Differences are whether RL13 & UL128 are mutated (-) or wildtype (+), and whether RL13 and/or UL131A have tet repressors in front of them.

We have variants with no tags, or with a P2A-GFP after IE2. This produces detectable GFP by 48 hrs post-infection (PI).

The RL13 mutation is a frameshift due to an extra A in the A tract 168bp after the ATG.

The UL128 mutation is a premature stop codon due to a G>A mutation following this sequence: TCCAGATTGATCCATC

BAC	Derived virus	RL13	UL128
'Vanilla' BACs containing no tags			
pAL1111	RCMV1111	–	–
pAL1119	RCMV1119	+	–
pAL1120	RCMV1120	–	+
pAL1128	RCMV1128	+	+
pAL1789	RCMV1789	-	+ (G>T mutation)
With IE2-P2A-eGFP			
pAL2159	RCMV2159	–	–
Tet repressible BACs containing no tags			
pAL1778	RCMV1778	-	+ (tetO)
pAL1516	RCMV1516	+ (tetO)	-
pAL1502	RCMV1502	+ (tetO)	+ (tetO)
Tet repressible BACs with IE2-P2A-eGFP			
pAL2167	RCMV2167	-	+ (tetO)
pAL2160	RCMV2160	+ (tetO)	-
pAL2157	RCMV2157	+ (tetO)	+ (tetO)

General Considerations

The BACs are approx 250kbp, and are 1 copy/cell in E.coli, so the main differences to 'normal' plasmids are that you have to be careful not to shear the DNA, and maxiprep yields are a lot lower. We keep the DNA in the fridge, not the freezer – freeze thawing tends to shear the DNA.

Transfecting DNA into E.coli

If transfecting BAC DNA into competent bacteria, any standard cloning strain will do. Due to the size of the BAC, electroporation will give much better results than heat shock. The BACs are all chloramphenicol (12.5μg/ml) resistant.

If you got the BACs from us as agar stabs in SW102 bacteria, then these must ALWAYS be grown at 32°C. They are recombineering bacteria, the recombineering genes are turned on at higher temperatures, and if they are turned on for an extended period the bacteria die.

Maxiprepping BACs

1. Pick a single colony in the morning & inoculate into 5ml LB/Chlor (12.5μg/ml).
2. Grow during the day in a shaking incubator. This is your starter culture. If you don't do a starter culture during the day your DNA yields will be lower.
3. Prepare flasks of 500ml LB/Chlor (12.5μg/ml) (1 flask for each BAC you are growing). For good aeration the flask should be 4x the volume of media.
4. Late in the afternoon, pour the whole 5ml of the starter culture into the 500ml LB and grow overnight in a shaking incubator.
5. The next morning, maxiprep the DNA. In theory you can use any maxiprep kit, however we've had best results with the Nucleobond BAC 100 kit. The main difference to standard kits is that the lysate from alkaline lysis are filtered by filter paper rather than syringe or centrifugation. This is both kinder to the large BACs, and faster than centrifuging.
6. The kit offers a BAC method – this should be followed. Important differences to the standard protocol are the use of double volumes for the alkaline lysis (24ml vs 12ml) to compensate for the larger bacterial pellet, and the use of filtration to clarify the supernatant. Prior to filtering the lysates, the manual gives you the option of doing a short & slow centrifugation step. We recommend doing this - it increases your DNA yields. If you don't do it then a lot of the supernatant stays complexed with the cellular debris, and never goes through the filter paper. We centrifuge for 15min, 6000rpm in the same pots that the bacteria were initially spun down in.
7. At the very end of the protocol, resuspend the pellet in 100μl Tris or TE. Concentration should be 100-300ng/μl.

Recovering Virus from the BAC

UL128-/RL13- BACs

If the BAC is mutated for both RL13 and UL128, then recover in fibroblasts. Any transfection method should work but the BAC is big, and fibroblasts do not transfect well, so you don't get many plaques. We've used effectene (qiagen) (transfect in a T25, using the protocol for 6cm dishes) but get more plaques with an Amaxa Nucleofector & the basic fibroblast kit. We use program T-16, 2μg DNA and 1×10^6 cells. Sometimes it's necessary to add more cells the next day. We get 10-20 plaques from this, and they should be visible by ~10 days. Once you get plaques, it speeds things up if you trypsinise the cells & reseed them into the same flask. This just spreads the infected cells out, so they can start new plaques. Once the virus is going, trypsinise & re-seed the same flask twice a week until you get 100% CPE. Supernatant from a T25 gives enough virus to infect 5-10 T150's.

RL13+ BACs

We don't recommend you use a BAC in which RL13 is intact – it is virtually impossible to recover an RL13 intact virus from an RL13 intact BAC, the gene always mutates. If a RL13+ BAC must be used, you'll need the tet-repressible version (see below).

UL128+ BACs

As with RL13, we recommend using one of the tet regulated UL128 BACs, rather than the BACs where UL128L is constitutively repressed (see below). However if you want to use one where UL128L is expressed constitutively, these are recovered fastest by transfecting into fibroblasts. UL128L will mutate after a while if grown in fibroblasts, although you may be able to get some virus grown before that happens. Titres are also very low ($<5x10^5$ pfu/T150).

If you are worried about mutation in UL128L, you can consider passaging virus in cells where gH/gL/UL128L is required (e.g. HUVECs/ARPE19's/RPE-1). We've tested ARPE19's but titers are poor - $<5x10^5$ pfu/T150. The virus grows faster & gives higher titres in RPE-1's (Clontech/ATCC) than in ARPE19's. We have seen some viruses mutate in UL/b' when passaged in RPE-1 cells (see Murrell et al, 2016), so be aware of this risk. If you infect RPE-1 cells at very low MOI, you will need to trypsinise the cells weekly & re-seed them into fresh flasks, in order to spread out the infected cells to start new plaques - the virus does not spread well through the supernatant.

Tet repressible BACs

These BACs have tet operators in front of RL13 and/or UL131A. If the tet repressor binds to the tet operators, transcription is inhibited. If there is no tetR present, transcription occurs as normal. We have made a HFFF cell line that expresses the tetR; if you recover virus from the BAC in this cell line you get good titres and there is little risk of mutation occurring. It's by far the easiest way of working with BACs containing RL13 and/or UL128. Although genetically intact, the virus produced will however lack gpRL13 and/or gpUL128-gp131A in the virus envelope. See below for methods to recover phenotypically wildtype virions.

HFFF-Tet cells

These HFFFs have been immortalised with htert (Hygromycin selection) and express the Tet repressor (Puromycin selection, selected in 1µg/ml). The tetR contains an NLS & is expressed from the retroviral LTR, puro is expressed from an IRES after tetR. We don't maintain them in selection, and have never had any issues with them losing Tet repressor expression. They are grown & used the same as any HFFF's – we use DMEM with 10% FCS.

Growing tet-repressible BACs

This is essentially the same as growing a UL128-/RL13- virus. Just transfect, trypsinise & reseed twice per week, & let the virus go through the cells, then recover supernatant. As with the UL128-/RL13- virus, supernatant from a T25 should give enough virus to infect >10 T150's.

Although RL13 and UL128 are repressed in the HFFF-Tet cells, the repression is not complete. It's probable that as the virus grows some expression breaks through. You will probably find that the speed of growth & level of cell free virus follows this kind of pattern:

RL13-/UL128- > RL13+ > UL128+ > RL13+/UL128+

Recovering wildtype virions

After you've grown virus in the HFFF-Tet cells you should have a nice high titre of virus that is genetically wildtype, but lacks gpRL13/gpUL128 in the virion. To recover phenotypically wildtype virus, you will need to do a single passage in normal cells. The only cells this virus will infect are HFFF, however titres are better from RPE1 than HFFF. We therefore do a high MOI infection of HFFF, then co-culture the HFFF with RPE1s at a ratio of ~1 HF:5 RPE1. The virus will transfer into the RPE, then trypsinise & reseed weekly (without adding any fresh cells) to get the virus to spread through the RPE1 cells. Once you have 100% CPE, harvest virus from the supernatant.