# D68EV3C large scale purification

**PAGE24-00037**

Author: **Wang, Korvus**
Date Started: **2024-Jan-10**
Experiment Started:
Projects: **Purification**;**ASAP**
Related Pages: **PAGE23-01458**
Referenced by:
Tags:

---

## Notes and Thoughts from the last large scale...

### Potential ways to remove DNA:

- PEI
need to check if there are clusters of negatively charged region in the protein - matlab bioinf toolbox
Doesn't look like it
Is the protein DNA binding?
No, 3C is a protease
Only concern is target is slightly negativly charged overall (PI=7.21) at HEPES buffer pH (7.4)
- DEAD52
Ask Mike if he knows where it is

- Benz soak
After loading onto column, wash with binding buffer. Soak resin in buffer with 1:4000 benz, 2mM $MgCl_2$, 150-200mM NaCl depending on what the protein can take. Leave capped in cold room for about 1hr, then wash with high salt buffer.
Other alterations:
Maybe raise the pH of the buffer to around 8 for IMAC, and back down to 7.4 during gel filtration
This might help with the precipitation issue as welll

Protein has two free cysteines, which may cause homodimer formation during purification (need more TCEP?)

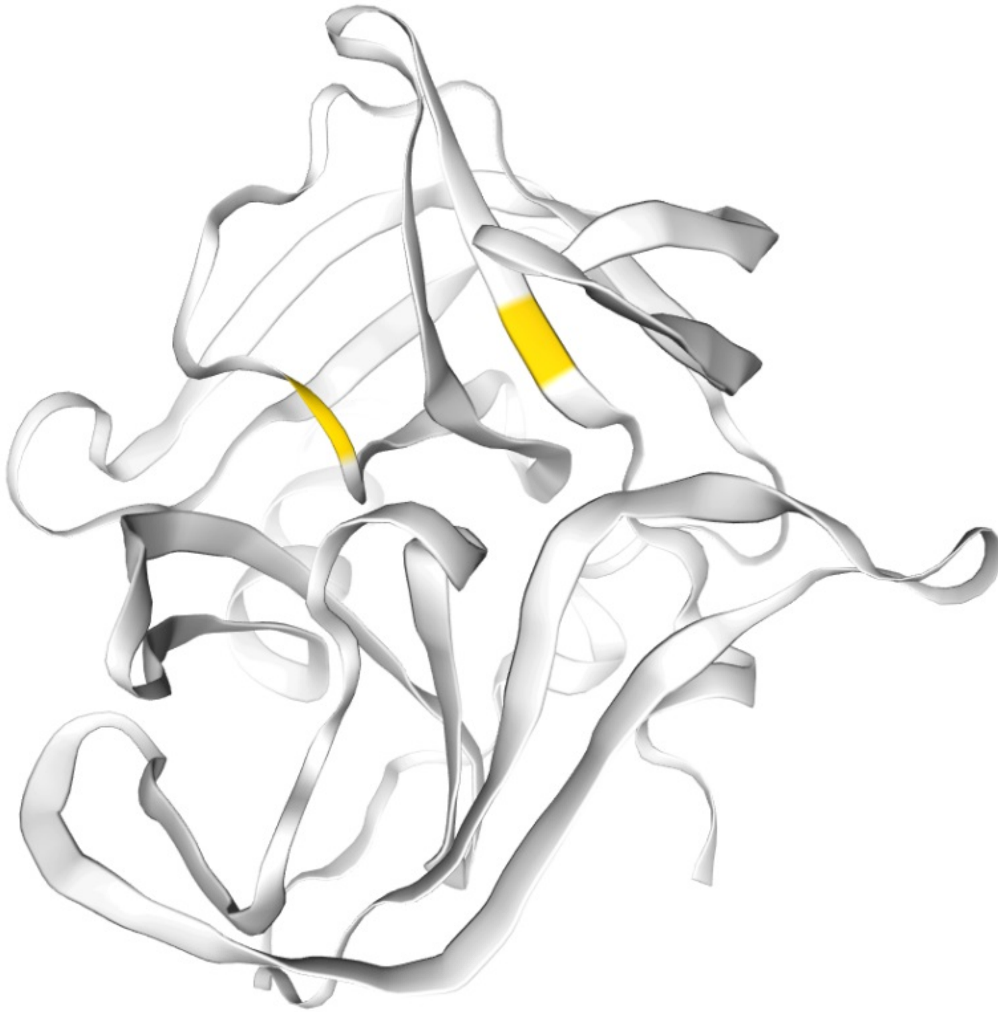
---

## D683C sequence analysis

```
  1  MGPGFDFAQA IMKKNTVIAR TEKGEFTMLG VYDRVAVIPT HASVGEIIYI NDVETRVLDA
 61  CALRDLTDTN LEITIVKLDR NQKFRDIRHF LPRCEDDYND AVLSVHTSKF PNMYIPVGQV
121  TNYGFLNLGG TPTHRILMYN FPTRAGQCGG VVTTTGKVIG IHVGGNGAQG FAAMLLHSYF
181  TDTQKHHHHH H
```

---

## SwissModel showing cysteine locations



**IMAC**

**----------------------------------------**
**Expression ID: D68EV3CPROA-e006 (Nathan)**
**Purification ID: D68EV3CPROA-p007**
**---------------------------------**

**D68EV3CPROA-c001**

MGPGFDFAQAIMKKNTVIARTEKGEFTMLGVYDRVAVIPTHASVGEIIYINDVETRVLDACALRDLTDTNLEITIVKLDRNQKFR
DIRHFLPRCEDDYNDAVLSVHTSKFPNMYIPVGQVTNYGFLNLGGTPTHRILMYNFPTRAGQCGGVVTTTGKVIGIHVGGNGA
QGFAAMLLHSYFTDTQKHHHHHH

21283.3 Da

10430 mM-1cm-1

PI=7.21

**Buffers:**
Lysis buffer - 50 mM HEPES pH 8.0, 500 mM NaCl, 5% glycerol, 0.5mM TCEP, 20mM imidazole
Wash buffer 1 - 50 mM HEPES pH 8.0, 100 mM NaCl, 5% glycerol, 0.5mM TCEP, 2mM MgCl, 1:2000 benzonase
Wash buffer 2 - 50 mM HEPES pH 8.0, 500 mM NaCl, 5% glycerol, 0.5mM TCEP, 30mM imidazole
Elution Buffer - 50 mM HEPES pH 8.0, 500 mM NaCl, 5% glycerol, 0.5mM TCEP, 500mM imidazole
Gel Filtration Buffer (SEC) - 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 0.5mM TCEP

Expression was carried out by Nathan Wright in bubble columns. 10L culture total. 244g pellet harvested.

1. 244g of pellet was thawed and resuspended with enough lysis buffer so that the final volume is 2L. Supplement with 1:4000 dilution of benzonase, 1mg/mL lysozyme, and 2mM MgCl.Incubate for 30min at RT.

1. Sonicated on ice at 50% amplitude for a total of 7-minute sonication time (4 seconds on 12 seconds off) with thick probe. Sonication done in 2 batches.
2. Clarified lysate by centrifugation at 38,000rpm, 4°C for 1 hour. Used JLA16.250 rotor, done in 2 batches. Supernatant poured into 2L beaker.

**IMAC**

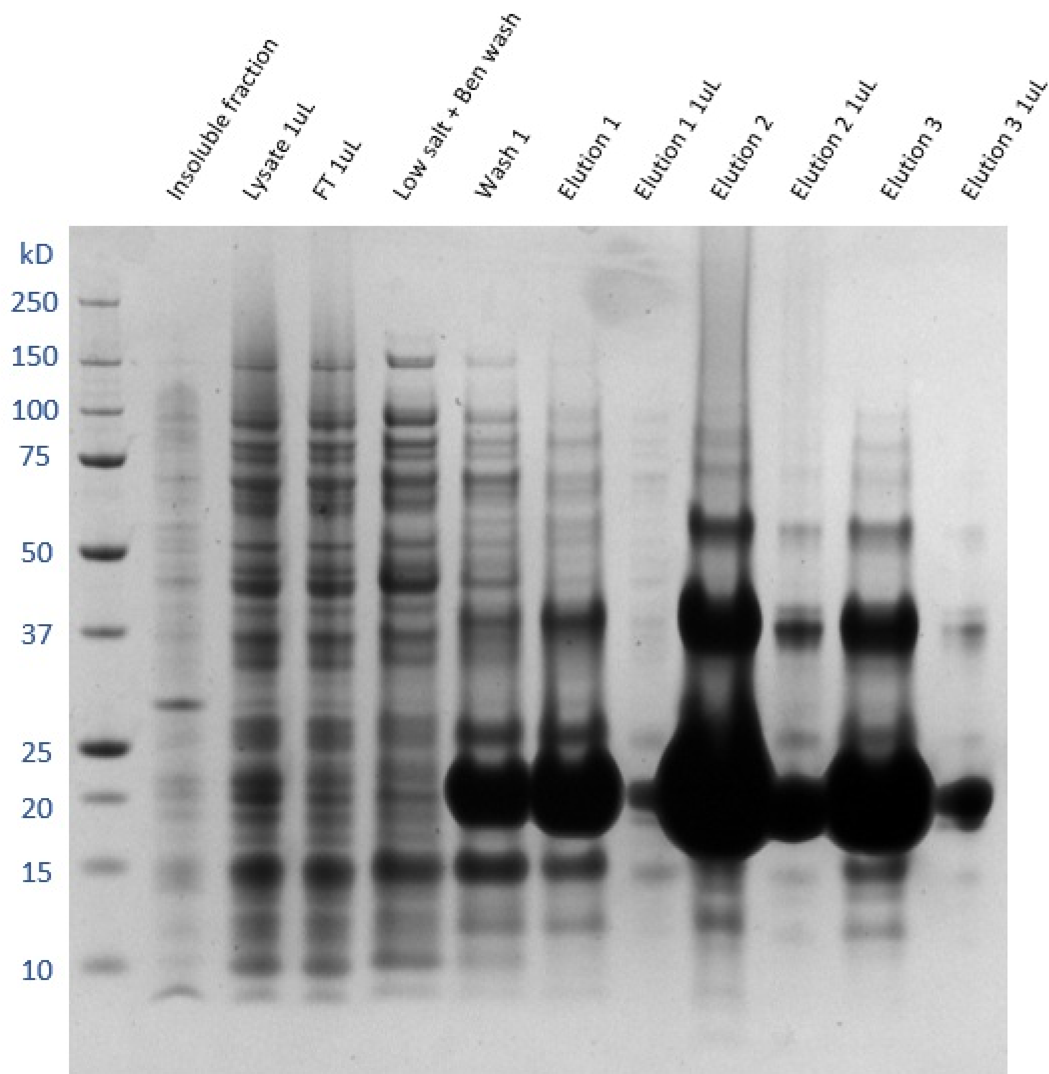
1. Wash and equlibrate 30mL bed volume of Ni Sepharose resin on large gravity flow column, first with distilled water, then with wash buffer.
2. Put stopper on column. Re-suspend resin with small volumes of the lysis buffer, and pour into beaker with lysate. Stir in cold room gently for 30min.
3. Pour mixture back onto gravity flow column and let flow through.
4. Add 200mL of wash buffer 1. Cap the column and leave rotating for 30min to digest remaining DNA. Drain wash buffer 1 and add 200mL wash buffer 2. Allow to flow through.
5. Elute with 30mL elution buffer, 3 elutions carried out
   Elution 2 started to precipitate mid-elution!! perhaps because concentration was too high? Immediatly diluted with 20mL of base buffer and the precipitants appear to have re-solubilised.
   Made all the elutions up to 50mL to prevent them from crashing overnight.

**68**
E1: 1.16 mg/mL
E2: 6.22 mg/mL
E3: 3.24 mg/mL

A260 looks good across all elutions. DNA issue resolved.
Surprisiungly lower than expected, since elution 2 was crashing out?

---

**IAMC results**



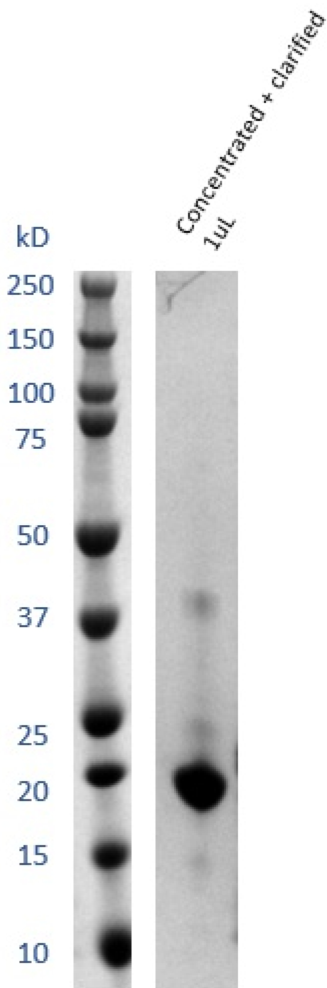

---

**Sample concentration for gel filtration**

Elutions pooled and diluted to 500mL with base buffer to reduce the final [imidazole] after concentration
Attempted to concentrate with Vivaspin 50 (10kDa MWCO) tangential flow concentrators.
Sample spurted everywhere at one point
Also the peristaltic pump generates a lot of heat and kind of cooked some of the protein.
Ended up using 3 cassettes and had to bury them in ice. Still a thick layer of jelly-like precipitated protein on top.
After reaching ~90mL, stopped concentration. Spun samples down at 20,000xg in JLA25. Ran sample and actually looks like most of what crashed out was the contaminants?

---

**Concentrated sample**




---

**Gel filtration run**

Decided to use XK 50/100 Superdex 200 pg column. 2L bed volume and loads up to 30mL sample. 500mg sample load can acheive analytical resolution.
MAKE SURE TO FLANGE ALL TUBINGS DUE TO HIGH PRESSURE

Run 30mL first to check if sample crashes at pH 7.5.
Loaded sample using 50mL superloop, prepared using the following video as guide
https://www.youtube.com/watch?v=Eavlht55bq0
Collect 35mL fractions with falcon tubes in the fraction collector (6 casettes). Start collection after 0.4CV (anything before 0.3 is void)

All looks good. No pressure change which indicated no significant precipiration. Sample came out near the end. Sample falcon tubes ran out near the end but is fine cuz all the peaks already came out.

Further concentrated the remaining samples down to ~45mL. Clarified by centrifugation at 20,000xg and injected via 0.2uM syringe filter.

Also ran fine. Peak profile looks similar to previous run.

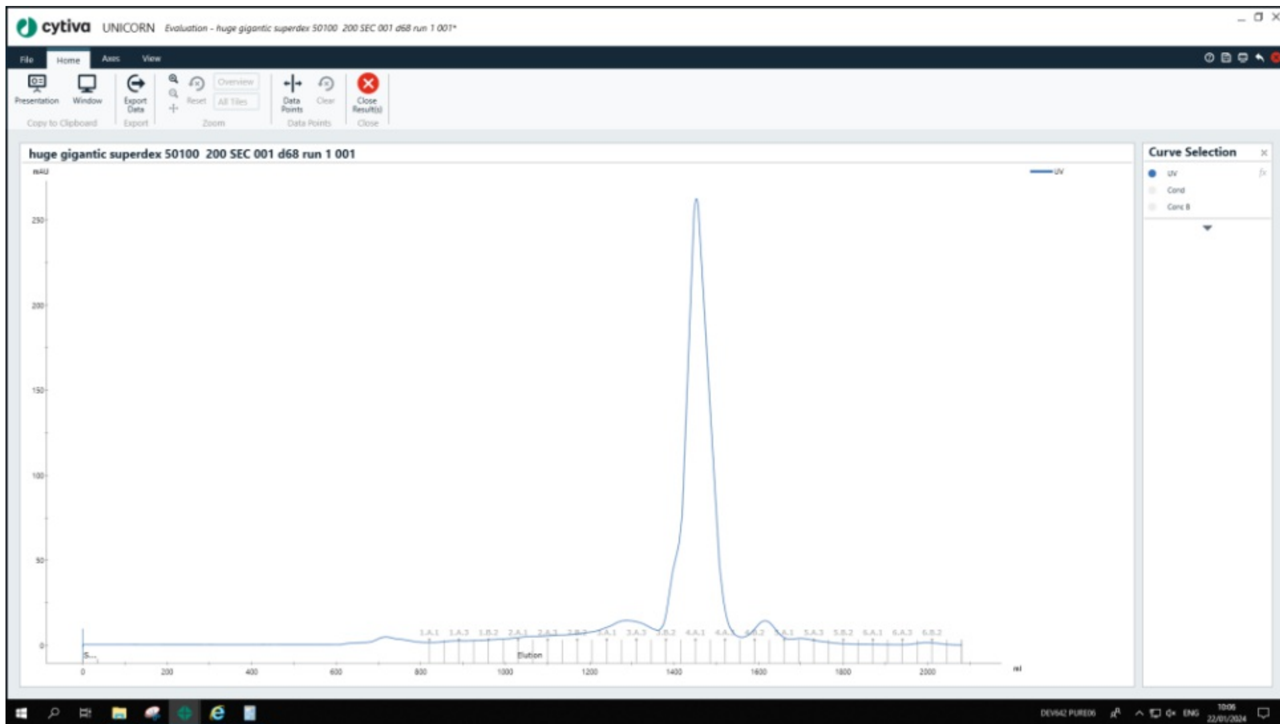
Run 1 Fractions:
3B3: 1.74 mg/mL
4A1: 2.25 mg/mL
4A2: 0.58 mg/mL

Run 2 Fractions:
3B2: 2.68 mg/mL
3B3: 5.26 mg/mL
4A1: 2.73 mg/mL
4A2: 0.56 mg/mL

Fractions ran on gel and concentrated in Amicon 10kDa MWCO concentrators.

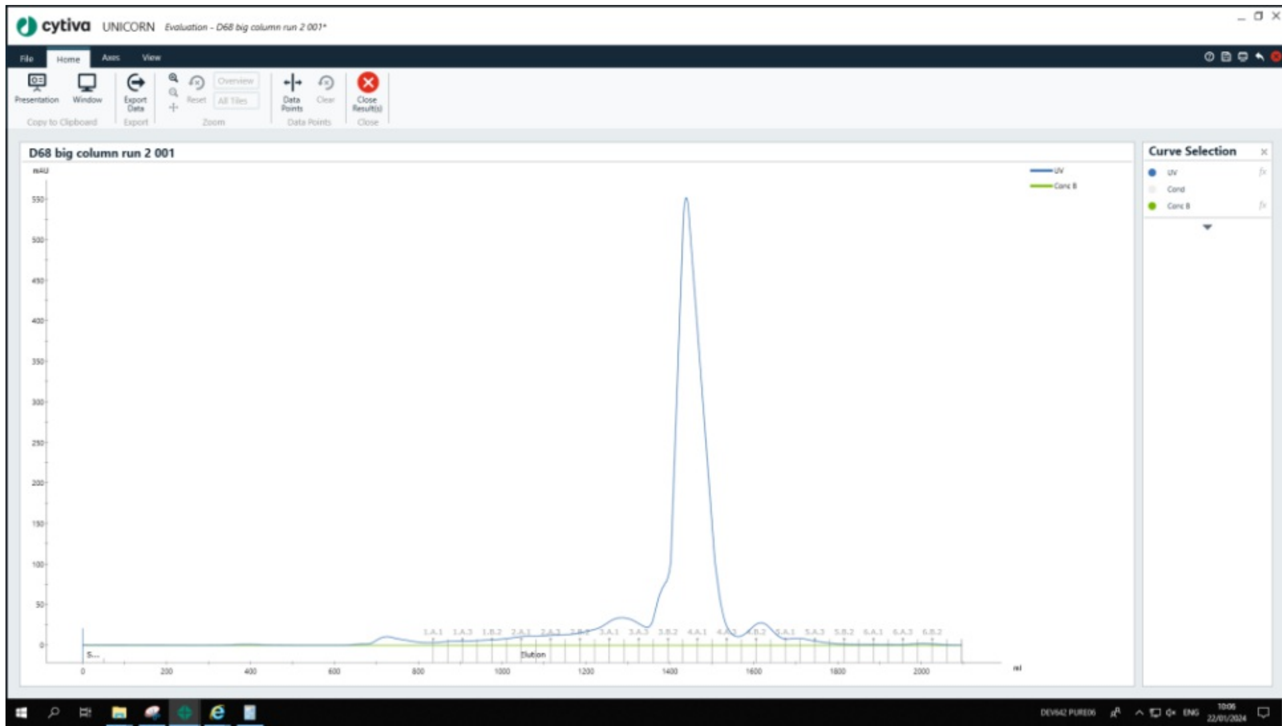
---

**Run 1**

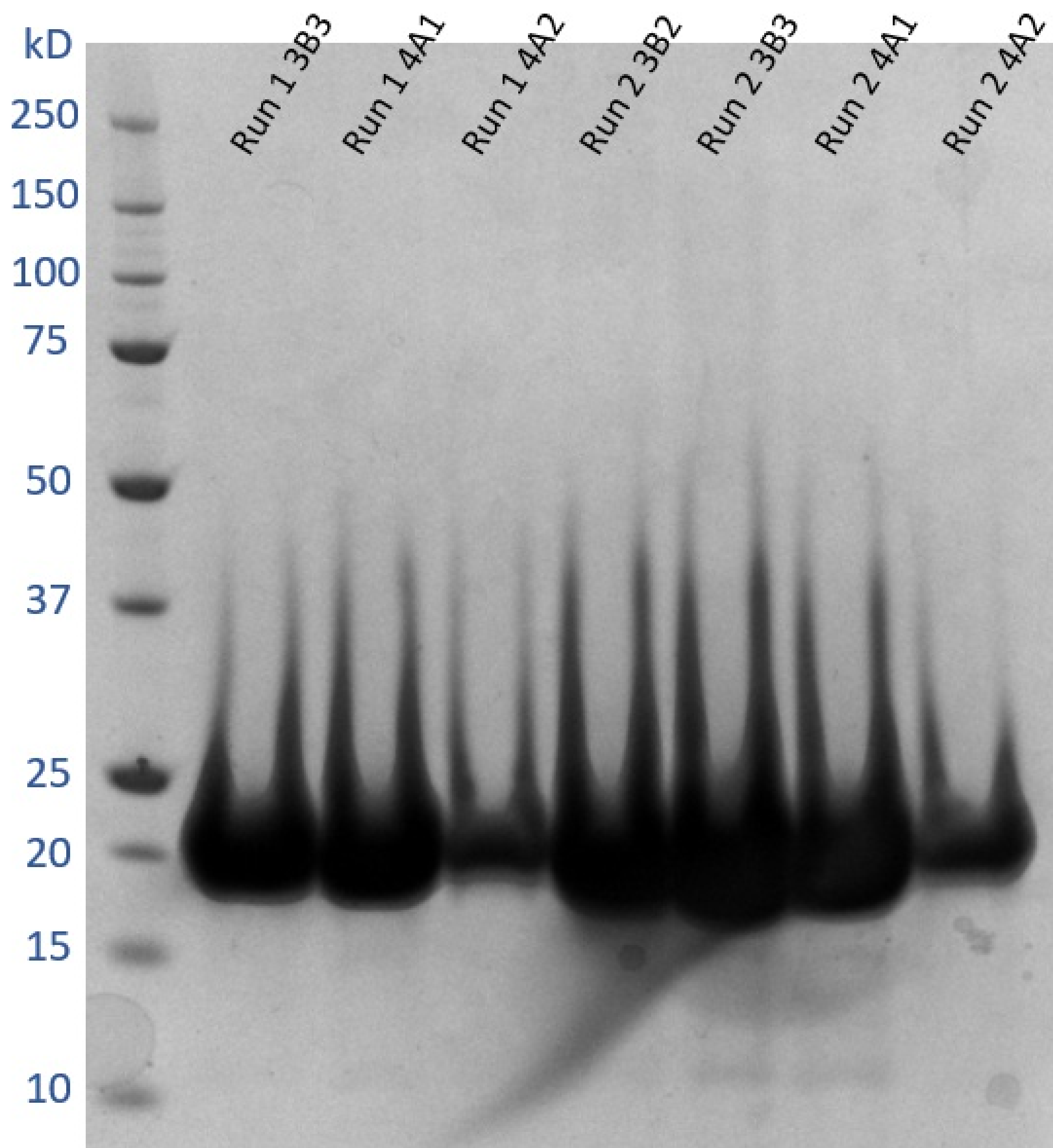



---

**Run 2**




## SEC results




---

**Final sample**

**Final sample:**
22.54 mg/mL
241x100uL
543.2 mg total

NEED FINAL SAMPLE GEL

Previous purificaition (p004) got 128.3mg from 2L.
Expected from 10L: 128.3x5 = 641.5mg
Achieved 84.5% of expected yield
probably lost some during initial concentration

Conclusion: scale-up is fairly comparable to original shake-flask method..

**RapidFire**

# D68EV3CPROA-p007

Good mass!!