Protein Production UK

# Protocol for baculovirus expression and purification of proteins in insect cells

## 1. Cell Maintenance

Passaging cells

Passaging of cells is required every 3-4 days. New cells should be thawed once passage is higher than ~p25.

1. Take 10 µl of ExpiSf9 cell stock from a shake flask and pipette into an Eppendorf.
2. Add 10 µl of trypan blue, mix well and pipette into a reservoir of a LunaII Cell Counting slide.
3. Count the cells and check viability. Viability should be maintained at >90%.
4. 4. Calculate the ratio of cell stock to ExpiSf CD Medium needed for a given dilution (cells are often split to $5x10^5$ cells/ml every 3-4 days).
5. Pipette the media and cell stock into a new sterile flask and incubate at 27°C, 120rpm.
6. Record the passage number on the flask (add 1 to the number on the feeder flask).

NB. If running transfection or infection experiment, it is best practice to set up cells at $8x10^5$ cells/ml the day before.

Freezing cells

1. Spin the cells at 100g for 5-10 minutes and remove the supernatant.
2. Resuspend cells in the volume to give $12x10^6$ cells/ml in Bambanker or use a mix of 70% ExpiSf CD Medium/20% FBS/10%DMSO.
3. Add 1 ml of suspension to each cryovial.
4. Either freeze in a Mr. Frosty container (Nalgene) or place straight into -80°C but this does kill more cells (slower freezing, moving from 4°C to -20°C to -80°C over few hours or days is better.)
5. Either keep in the -80°C freezer or transfer to liquid nitrogen for long term storage.

Thawing cells

1. Prepare 25 ml of ExpiSf CD Medium in a 125 ml shake flask.
2. Rapidly thaw 1 vial of cells in a water bath at 37°C.

3. Resuspend thawed cells gently into room temperature ExpiSf CD Medium and add all the thawed cells to the prepared flask (~5x$10^5$ cells/ml)4. Incubate at 27°C, 120rpm.

5. When the culture reaches a density of around 2x$10^6$ viable cells/ml, passage the cells (should take about 5 days).

Passage the cells at least two times before using in other applications.

## 2. Preparation of linearized bacmid

Baculovirus construction is based on the protocol of Zhao et al (DOI: 10.1093/nar/gng006) The mCherry containing baculovirus bacmid was kindly provided by Arnaud Poterszman (doi.org/10.1038/s41598-021-04715-5).

Extraction of bacmid using the Nucleobond BAC 100 Kit 4

1. Inoculate 200 ml of LB + Kan (50 ug/ml) + Cm (25 ug/ml) with mCherry bacmid glycerol stock.
   NB:: If starting from bacmid DNA, transform into any *E. coli* cloning strain and plate out onto agar plates containing Cm and Kan: expect transformation frequency of the large bacmid to be very low.
2. Grow O/N at 37°C.
3. Harvest bacteria from an LB culture by centrifugation at 4,500–6,000 x g for 15 minutes at 4°C.
4. Carefully resuspend the pellet of bacterial cells in 24 ml Buffer S1 + RNase A.
5. Add 24 ml Buffer S2 to the suspension. Mix gently by inverting the tube 6–8 times.
6. Incubate the mixture at room temperature for 2–3 minutes (max. 5 minutes). Do not
7. vortex, as this will release contaminating chromosomal DNA from cellular debris into the suspension.
8. Add pre-cooled 24 ml Buffer S3 to the suspension. Immediately mix the

lysate gently by inverting the flask 6–8 times until a homogeneous suspension containing an off-white flocculate is formed. Incubate the suspension on ice for 5 minutes.

9. Spin lysate at 4000 rpm for 15 minutes.
10. Equilibrate a NucleoBond BAC 100 (Maxi) column with 6 ml Buffer N2. Allow the column to empty by gravity flow. Discard flowthrough.
11. Place a NucleoBond® Folded Filter in a funnel of appropriate size. Wet the filter with a few drops of Buffer N2 and load the bacterial lysate onto the wet filter and collect filtrate.
12. Load the cleared lysate from step 9 onto the NucleoBond Column. Allow the column to empty by gravity flow.
13. Wash the column twice with 18 ml Buffer N3. Discard flowthrough each time.
14. Elute the plasmid DNA with 15 ml Buffer N5.

    NB: Preheating Buffer N5 to 50 $^{0}$C prior to elution may improve yields for high-molecular weight constructs such as BACs.
15. Add 11 ml isopropanol to precipitate the eluted plasmid DNA.
16. Mix carefully and centrifuge at ≥ 5,000 x g for 30 minutes. Carefully discard the supernatant.
17. Add 2 ml 70 % ethanol to the pellet. Vortex briefly and centrifuge at ≥ 5,000 x g for 10 minutes at room temperature. 2 ml
18. Carefully remove ethanol from the tube with a pipette tip. Allow the pellet to dry at room temperature no less than the indicated time. Drying for longer periods of time will not harm the quality of plasmid DNA but over-drying may render the DNA less soluble.
19. Dissolve pellet in 300 ul sterile deionized $H_2O$. Expect yield of approximately of 180 ug (600 ng/ul).

<u>Bsu361 digestion</u>

1. Digest 10 x 6 ug aliquots of the bacmid each in 100 ul final volume containing 10 ul 10 X NEB buffer 3 and 1 ul 100X BSA 1 µl Bsu36I.
2. Incubate for 2h at 37°C.
3. Add another 1 µl Bsu361 per 6 µg of DNA.
4. Incubate another 2-3 hours at 37°C.
5. Heat at 72°C for 20 minutes. No additional purification of the bacmid is required.

6. Divide into 10 ul aliquots in PCR tubes and store the cut bacmid at -70°C.

## 3. Construction of baculoviruses

Production of P0 virus

Prepare ExpiSf9 cells at 5-7x$10^5$ cells/ml.

| **Reagent** | **24 well plate** | **12 well plate** | **6 well plate** |
|---|---|---|---|
| ExpiSf9 Cells at 5-7x$10^5$ cells/ml | 500 µl | 1000 µl | 2000 µl |
| **Transfection Mix (per well)** | | | |
| ExpiSf CD Medium | 50 µl | 100 µl | 200 µl |
| Linearised Bacmid | 250 ng | 500 ng | 750 ng |
| Vector | 100 - 500 ng | 200 – 1000 ng | 500 – 1500 ng |
| FugeneHD | 1.5 µl | 2 µl | 4 µl |

1. Add the necessary volume of ExpiSf9 cells at 5-7x$10^5$ to the chosen culture plate, as indicated in the table above. One well is required per vector, plus two control wells (cells only and a well to be transfected with GFP).
2. Leave cells to attach for 30 minutes to 1 hour at room temperature.
3. Prepare transfection mix for each vector in an Eppendorf, according to the concentrations above. A control with GFP should be included here.
   For 24 well plates:
   Gently mix 5 µl Bacmid (approx. 50-60 ng/µl), 100-500 ng Vector DNA and 50 µl ExpiSf CD Medium. Then add 1.5 µl FugeneHD, pipetting directly into the liquid.
   NB: Avoid pipetting against the plastic as this may reduce transfection efficiency.
4. Mix well and incubate for 30 minutes at RT.
5. Add transfection mix slowly (to avoid disrupting the monolayer) to cells in the appropriate well.
6. Gently swirl the culture plate to distribute the transfection mix across the well.
7. Incubate static for 6-7 days at 27°C (check GFP control for expression to make sure the transfection has worked).

8. Harvest cells into eppendorfs. Centrifuge at 6,000 x g.
9. Collect viral supernatant and store in at 4°C in the dark. THIS IS YOUR P0 VIRUS STOCK.
10. Discard the culture plates.

Production of P1 virus

Although expression screening can be performed using the P0 virus if time is an issue, the most reliable route is to first amplify the P0 virus and then use the resulting P1 virus for small or medium scale expression screening.

1. Infect 3 mL of ExpiSf9 cells at $1x10^6$ cells/ml with 10 µl of P0 stock (1:300 dilution) in 24 well round bottom blocks
2. Incubate for 6-7 days at 27°C, 220 rpm.
3. After 7 days post-infection, check for expression.
4. Centrifuge blocks at 1,000 x g for 10 minutes at room temperature, to pellet the cells and debris.
4. Collect viral supernatant in fresh tubes and store at 4°C in the dark. THIS IS YOUR P1 VIRUS STOCK.

## 4. Small-scale expression screen

Infection with P1 virus

1. Prepare 24 deep well round bottom plates with 3 ml of ExpiSf9 cells at 1x106 cells/ml in each well.
2. Add 30 µl P1 virus (1:100 dilution) to each well (including a GFP control virus) and incubate for 3-4 days at 27°C, 220 rpm.
3. Transfer 3 ml to a 24-well deep well plate with conical bottoms and centrifuge for 15 minutes at 6,000 x g, remove the supernatant and freeze the cells at -80°C for at least 30 minutes.

Small-scale Protein Purification using NEBExpress Ni-NTA Magnetic Beads Kit

Lysis Buffer: 20 mM Sodium Phosphate, 300 mM NaCl, 10 mM Imidazole, NP40 to 1% final concentration, pH 7.4, cOmplete Protease Inhibitor (1 tablet/50ml) (Roche 04693116001)
Wash Buffer: 20 Mm Sodium Phosphate, 300 mM NaCl, 20 mM Imidazole, pH 7.4
Elution Buffer: 20 mM Sodium Phosphate, 300 mM NaCl, 500 mM Imidazole, pH 7.4
NB: After preparing, keep all buffers cool on ice where possible

Single Tube Format (1.5 ml eppendorfs)

1. Dispense 50 µl of bead slurry into each tube, and add 200 µl lysis buffer to equilibrate beads
2. Place tube onto a magnetic rack to pellet the beads, remove and discard supernatant.
3. Resuspend thawed cell pellets in 1 ml lysis buffer. This is your lysate. Keep 10 µl lysate sample for analysis.
4. Centrifuge at 14,000 rpm, 4°C for 10 minutes
5. Add 1 ml lysate to equilibrated beads.
6. Incubate for 30 minutes with end-over-end mixing at 4°C.
   NB: Beads may adhere to sides or cap of the tube during mixing. Samples can be briefly spun in microcentrifuge prior to pelleting with magnetic rack.
7. Place tube in magnetic rack and remove supernatant. Keep 10 µl flowthrough sample for analysis.
8. Add 500 µl wash buffer and briefly mix beads, before returning tube to magnetic rack.
9. Remove supernatant and reserve 10 µl of wash sample for analysis.
10. Repeat wash steps (steps 8, 9) twice more, ensuring all wash buffer is removed in the final wash.
11. Add 100 µl elution buffer and mix for 2 minutes on a benchtop shaker at 850 rpm.
12. Place tube in magnetic rack to pellet the beads.
13. Transfer supernatant, containing eluted protein, to new tube. Keep 10 µl elute sample for analysis.
14. After completion, you should have 4 samples for each protein: lysate, flowthrough, wash, elute). Run an SDS-PAGE gel of samples to assess expression.

96-well Format

1. Resuspend bead slurry and dispense 50 µl into each well of a 96-well microtitre plate.
2. Place plate on 96-well microtiter magnetic separation rack and remove supernatant.
3. Add 200 µl lysis buffer to equilibrate the beads and mix briefly.
4. Place plate onto magnetic rack to remove supernatant.
5. Add 200 µl lysis buffer to the thawed cell pellets to resuspend, then add crude lysate to equilibrated beads. Keep 5 µl lysate sample for analysis.
6. Incubate for 30 minutes, 850 rpm, 4°C using a benchtop shaker.
7. Place plate on a magnetic rack and remove the supernatant. Reserve 5ul flowthrough sample for analysis.
8. Resuspend beads in 200 µl wash buffer and mix briefly.
9. Place plate on magnetic rack and remove supernatant. Reserve 5 µl as wash sample for analysis.
10. Repeat wash steps (steps 8,9) twice more, ensuring all wash buffer is removed in the final wash.
11. Add 100 µl of elution buffer and mix the suspension for 2 minutes on a benchtop shaker at 850 rpm.
12. Place plate on magnetic rack to pellet the beads.
13. Transfer supernatant, containing eluted protein, to a new plate. Keep 5 µl elute sample for analysis.
14. After completion, you should have 4 samples for each protein: lysate, flowthrough, wash, elute). Run an SDS-PAGE gel of samples to assess expression.

## 5. Scale up infection and expression

Production of P2 virus

1. Prepare 50 ml of ExpiSf9 cells at 1x $10^6$ cells/ml.
2. Add 500 µl P1 virus stock (1:100 dilution).
3. Incubate for 6-7 days at 27°C, 120rpm.

4. Transfer to a fresh, sterile Falcon and spin for 10 minutes at 1,000 x g. Discard the cell pellet.
5. Transfer the supernatant to a fresh 50 ml Falcon.
6. Filter sterilise (virus will pass through a 0.2 µm filter) and store at 4°C in a black falcon tube (or wrap the tube in foil). THIS IS YOUR P2 VIRAL STOCK.
7. For long term storage: add FCS to 10% (you can also freeze at -80°C or liquid N2)

Infection with P2 virus

1. Prepare 2.5 L of ExpiSf9 cells at $1x10^6$ cells/ml in a Thompson flask (or 5 x 500 ml in 2 L shake flasks).
2. Add 25 ml P2 virus stock (1:100 dilution) per 2.5 L (depending on the expression screen results).
3. Incubate for 3 - 4 days at 27°C, 120 rpm.
4. Take a 2 ml sample for small-scale analysis by Ni-NTA using the protocols in section 4.
5. Harvest by spinning at 6,000 x g for 15 minutes.
6. Freeze the pellet at -80°C ready for purification.

Large-scale Protein Purification

Lysis Buffer: 50 mM Tris pH 7.5, 500 mM NaCl, 30 mM Imidazole, 0.2 % Tween.

Ni Wash buffer: 50 mM Tris pH 7.5, 500 mM NaCl, 30 mM Imidazole.
Ni Elution buffer: 50 mM Tris pH 7.5, 500 mM NaCl, 500 mM Imidazole.
Gel Filtration Buffer: 20 mM Tris pH 7.5, 200 mM NaCl, (1 mM TCEP)

NB: All buffers should be filtered through a 0.22 µM filter. Buffers can be stored at room temperature, but should be cooled at 4ºC prior to use.

*Lysis*

Before starting lysis, begin equilibrating the AKTA Xpress system. Columns are stored in 20% EtOH, so should be equilibrated first into 0.22µM filtered dd$H_2O$, then into Gel Filtration Buffer using the 'Gel Filtration Equilibration' programme (Instant Run).

1. Take the thawed cell pellet and resuspend in ~50-100 ml Lysis buffer per 10 g pellet. Supplement this with 100 µl protease inhibitors (P8849, Sigma) and 5 µl Benzonase (0.562 mg/ml) per gram of cell pellet. (2mM $MgCl_2$ can be added to aid the Benzonase activity).
2. Make sure the lysate is homogeneous by stirring or pipetting up and down before proceeding onto the next stage. Keep lysate on ice.
3. Either:
    a. Pass the sample through a basic Z cell disruptor at 30 Kpsi
    b. Sonicate the sample for 3 x 3 minutes, using 9 second pulses at 60% amplitude. Swirl the sample after each 3 minute set.

NB: Sonication method is preferable due to the need to decontaminate following lysis. Keep lysate on ice following lysis step.

4. Centrifuge at 30,000 x g for 30 minutes at 4°C. Repeat this step if supernatant is still cloudy.
5. After centrifugation, the cleared lysate is decanted away from the insoluble fraction. Filter the cleared lysate through a 0.45 um bottle top filter. If the lysate quickly blocks the filter, centrifuge again. Do not proceed with the purification unless the product has gone through the filter.

*Purification using AKTA Xpress*

Initial purification should be completed at 4°C.

NB: This protocol uses IMAC/SEC to purify proteins, so the programmes specify for samples to be passed first over a Nickel Affinity (HisTrap) column, followed by a Size Exclusion column, before eluting fractions. Therefore, it is important that a HisTag is included in the initial constructs for expression.

1. Ensure an appropriate size exclusion column is connected to the AKTA system for the expected kDa of the protein.
2. Set up 'Gel Filtration Equilibrium' with Gel Filtration Buffer prior to starting lysis.
3. Run 'Mammalian Prepping System' (add 5ml nickel column here)

4. Run 'Mammalian Gel Filtration' (A2 line into sample here - use bung to stabilise line in plastic bottle)
5. Run 'Mammalian Cleaning System'
6. Run 'Gel Filtration Equilibrium' to put system & superdex column back into water
7. Run 'Gel Filtration Equilibrium' to put system & superdex column into ethanol overnight
8. Fractions should be picked for analysis on a gel based on blue peaks (UV)
9. Mix 10 µl of each fraction to be analysed with 10 µl of SDS-PAGE loading buffer in 96-well PCR plate.
10. Heat the samples at 95°C for 3 minutes.
11. Run gels for the samples using the appropriate markers.
12. Decide which fractions to pool together and combine for concentration (and subsequent cleavage, if necessary).
13. Measure the A280 using the Nanodrop UV spectrometer and calculate the concentration of protein in the solution.
14. The protein may need to be concentrated to around 2 ml before cleavage. Select an appropriate MWCO concentrator and pipette 2 ml of gel filtration buffer into Centrifuge for 1 minute at 4000 x g, then remove the buffer from the upper and lower chambers (this prepares the concentrator for use and reduces the chances of the protein sticking to the membrane).
15. Add your pooled protein fractions to the upper chamber of the concentrator. Spin for ~10 minutes at 4000 x g. Check the volume of sample.
16. Repeat until the target volume is reached.
17. Measure the A280 using the Nanodrop UV spectrometer and calculate the concentration protein in the solution.

<u>3C Proteolytic Cleavage</u>

1. Add ~50 µl of 3C Protease (1.957 mg/ml) per mg of protein sample. Incubate at 4°C for 1 hour, with end-over-end mixing. (For cleavage it can be helpful to have 1 mM TCEP present)
2. A gel can be run at this stage to check the cleavage has gone to completion. NB: If cleavage is not observed, incubate the sample overnight at 4$^{o}$C with end-over-end mixing until fully cleaved.

3. Pass the protein through a HisTrap column manually, collecting the flow through in 96 deep well plate.
   NB: This can be completed at room temperature, as the protein will elute quickly. At this stage, the cleaved protein is in the flowthrough.
4. Wash the column with 3 x 2 ml of wash buffer collecting the fractions.
5. Elute the protease and cleaved tag with 2 x 2 ml of Elution Buffer.
6. Run a gel of the cleaved and purified protein fractions
7. Decide which fractions to combine for concentration.
8. Measure the A280 using the Nanodrop UV spectrometer and calculate the concentration of protein in the solution.

Mass Spectrometry

Take 15 µl of sample at 20 µM concentration (µM = [conc in mg/ml]*1000/[MW in kDa]) and analyse using Mass Spectrometry.

Check that a peak is observed at the expected molecular weight for your protein.

AKTA Xpress Set Up

*Column Equilibration*

A1, A4: Gel Filtration Buffer

*Purification*

A1, A2: Ni Wash Buffer

A2: Sample (It will ask you to switch the line during the programme)

A3: Ni Elution Buffer

A4: Gel Filtration Buffer

F3: Empty Duran Bottle (This will be your flowthrough)

Column Position 1: 5ml Nickel Affinity Column (It will ask you to plug this in during the programme)

*Column Cleaning (SD200/SD75)*

A2: H2O, 20% EtOH

Columns should be stored in 20% EtOH.

Equilibrate and clean columns in the following order:

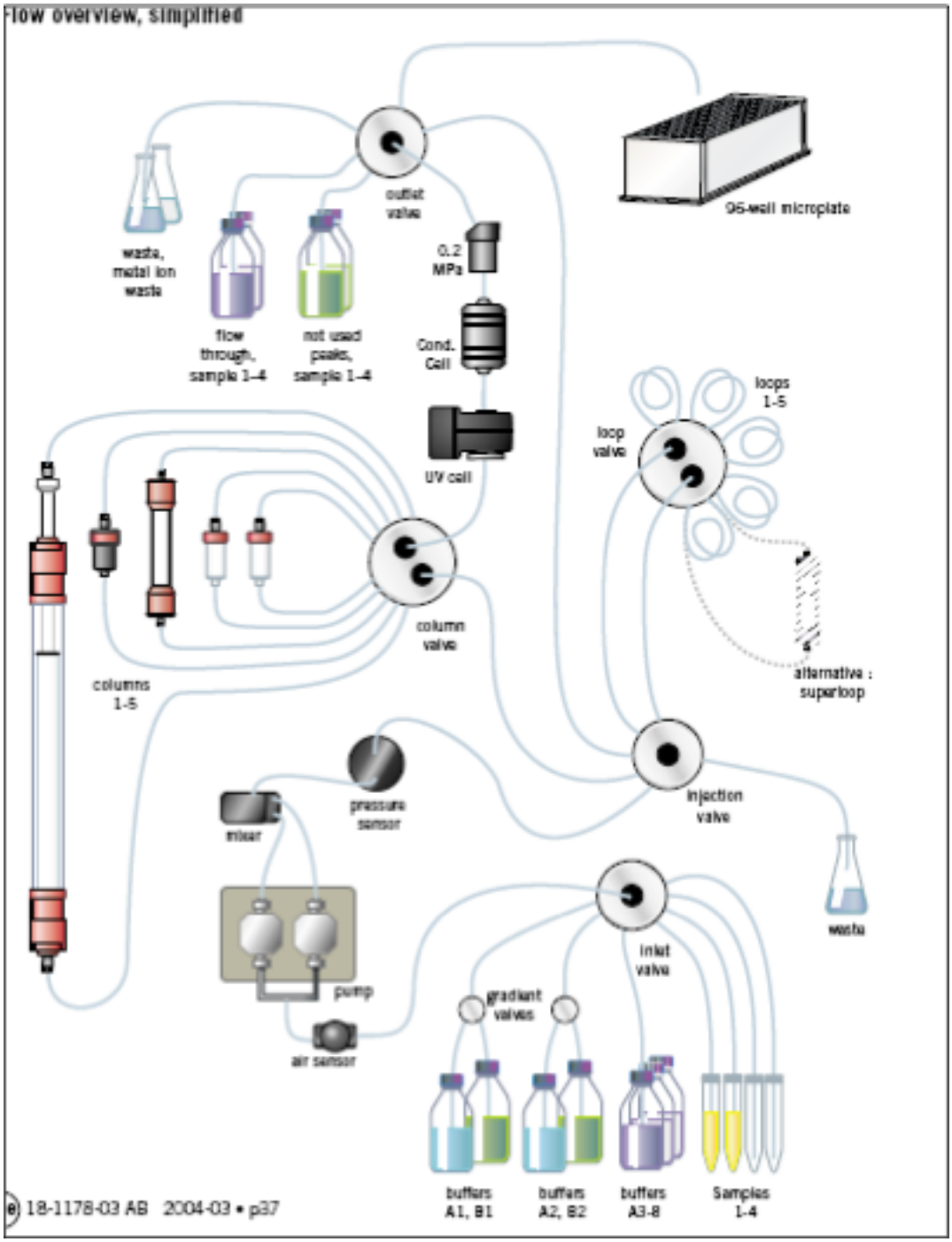
20% EtOH > H2O > Gel Filtration Buffer > H2O > 20% EtOH

*Column Cleaning (5ml Nickel Affinity Column)*

Regenerate columns with 5 column volumes of the following, using a peristaltic pump:

H2O > Stripping Buffer > H2O > 1M NaOH > H2O > 0.1M NiSO4 > H2O > 20 % EtOH

Stripping Buffer: 20mM $Na_2HPO_4$, 500mM NaCl, 50mM EDTA, pH 7.5


Flow overview, simplified
outlet valve
96-well microplate
waste, metal ion waste
0.2 MPa
flow through, sample 1–4
not used peaks, sample 1–4
Cond. Cell
UV cell
loops 1-5
loop valve
column valve
columns 1-5
alternative : superloop
pressure sensor
mixer
injection valve
waste
inlet valve
pump
gradient valves
air sensor
buffers A1, B1
buffers A2, B2
buffers A3-8
Samples 1-4
18-1178-03 AB 2004-03 • p37