Genotyping Protocols in General

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KAPA Mouse Genotyping Kits

Among multiple commercial kits that are specifically designed for genotyping, we specifically recommend this kit although this one is probably the most expensive.

Before switching to this kit, we experienced a lot of frustration due to frequent ambiguous assay results.
The ambiguity often appeared to come from excessive amplification of cross-contaminants, resulting in rather intense signals that are comparable to that of legitimate signals.
This happened when genotyping was carried out either by non-kit method or by a different kit (e.g., Phire Tissue Direct PCR Master Mix).
KAPA Mouse Genotyping Kit is *relatively* insensitive to trace amount of cross-contaminant, thereby faint signals due to cross-contaminats can be somehow distinguished from intense legitimate signals in most cases.
This, of course, is possible only when cross-contamination is kept minimal.

KAPA Mouse Genotyping Kit is not almighty, however.
Some genotyping assays may not yield clean publication-quality products when KAPA Mouse Genotyping Kit is used.
In such cases, other commercial kits (e.g., Phire Tissue Direct PCR Master Mix, or AccuStart II Genotyping Mix) may work better.

KAPA Mouse Genotyping Kits are manufactured by KAPA Biosystems.
Canadian distributor of their products has kept changing over years.
In 2017, we were purchasing KAPA Mouse Genotyping Kit from Sigma.
The price for this kit has changed rapidly as well, doubling in ~ 3 years.
Contact Craig Murray (craig.murray@sial.com) to find current situation.

There are two versions of KAPA Mouse Genotyping Kit: hotstart (HS) or non-hotstart.
At the Centre for Genome Engineering, we use hotstart version.
But for most applications, non-hotstart version works well.

The kits consist of two components:
- KAPA2G Fast HS Genotyping Mix
- KAPA DNA Extraction Buffer & Enzyme

The extraction buffer & enzyme are supplied in excess.
If you have enough of the extraction buffer & enzyme, you need to order only the PCR reaction mix (i.e., KAPA2G Fast HS Genotyping Mix).

Note: Genomic DNA prepared by KAPA Mouse Genotyping Kit can be amplified by most (if not all) other DNA polymerases without problem.

Ordering Information

There are multiple sizes available. The information shown below are limited to those we are using at the Centre for Genome Engineering.

KAPA Mouse Genotyping Kit HotStart, 500 x 25 uL rxns (PCR rxn plus DNA extraction)
Sigma, cat# KK7352, $809.00 (April 2017)

KAPA2G Fast HS Genotyping Mix (6.25 mL) (PCR rxn only)
Sigma, cat# KK5621, $303.00 (June 2017)

KAPA Mouse Genotyping Kit: DNA Extraction Protocol

Be careful to not introduce cross-contamination. Cross-contamination is one of major reasons we sometimes obtain ambiguous results. Even a supposedly negligeble amount of cross-contaminant can be disproportionally amplified to the levels which are comparable to legitimate samples. Use only filtered tips for everything.
Note that trace amount of cross-contamination may not be totally avoidable especially at the stage of harvesting ear notches. That's one big reason why we prefer KAPA Mouse Genotyping Kit.
Genomic DNA extracted by KAPA Mouse Genotyping Kit works best when PCR products are in the range of 150 bp ~ 1 kb. Although you may be able to detect up to ~ 5 kb sequence from DNA samples prepared by KAPA Mouse Genotyping Kit if a highly optimized condition is employed, salting-out method is far more suitable for such applications.

1. Transfer ear notch, or tail clip, to tube where extraction is to be done:

   method-1: extraction in PCR tubes
   Transfer ear tissue to bottom of PCR tube (8-strip is preferred), using a new tip for each sample.
   * Use pipette tip (attached to Pipetman) to do the transfer. Freshly prepared tissue sticks to tip very well. Old dried-up tissue can be hard to pick up.
   * Do the transfer on a sheet of white paper so that the sample can be spotted easily in case it falls off.
   * If a tube contains multiple pieces of tissues, transferring just one suffices. However, transferring all does not hurt either - essentially same results should be obtained.

   method-2: extraction in original microfuge-tube
   No transfer is required. This method is possible if you have two heat-blocks (75°C & 95°C).

2. Make Master Mix;

	50 ul rxn	*100 ul rxn*
Water	44 ul	88 ul
10 x KAPA Express Extract Buffer	5 ul	10 ul
1 U/ul KAPA Express Extract Enzyme	1 ul	2 ul

   * Ensure that the buffer is fully thawed and vortexed before use.
   * Be aware that precipitates form in 10x Extract Buffer during storage at -20°C. It probably does not affect assay. But just in case, disperse the pelletted precipitates after centrifugation and before taking up aliquot.
   * In most cases, 50 ul extraction solution per single ear clip suffices. For tail clip, ≥ 100 ul is preferred volume. You can use same 100 ul volume for tiny single ear clip, for large ear clip, for multiple ear clips, and for tail clip.
   * Be extra cautious to not introduce cross-contamination into main stocks of extract buffer, extract enzyme, and water. Use only filtered tips.

3. Add 50 ul, or 100 ul, of the Master Mix to each tube, using a new tip for each sample.
   * Make sure that sample is in solution. Spin briefly to bring sample into solution.
   * If sample is very small (- so small that only a close look can find it), volume may be reduced accordingly.

4. Extract genomic DNA by incubationg 15 min at 75°C, followed by 5 min at 95°C. Then, cool down.
   If samples are in PCR tube, use PCR machine with the following program;
   75°C, 15 min
   95°C, 5 min
   4°C

If samples are in original microfuge tubes, incubate 15 min in 75°C-heat block, followed by 5 min in 95°C heat block. Then, take out to cool down.

* Better use cap-closure to prevent "popping" which occurs during heating (especially at 95°C).

5. Vortex the samples a few seconds to help release more genomic DNA into solution.
   * The ear/tail tissue probably looks intact. Don't worry - DNA has been liberated. Vortexing will release more DNA into the solution.
   * If samples are in original microfuge tubes, vortexing can be done without problem, However, if samples have been transferred into PCR tubes, you need to devise some method. For example, you can use a full-skirted 96-well PCR plate to insert 8-strip PCR tubes, and use vortex machine equipped with adaptor for 96-well plate. Or, you could transfer the solution along with digested tissue into a new microfuge tube for vortexing purpose. Or, you just shake the 8-strips by your hand.
6. Centrifuge briefly to bring down splattered solution.
7. Store the samples at 4°C (up to 1 week) or at -20°C for longer period.
   * No need of separating solution from remains of digested tissues. Just centrifuge briefly before taking up aliquot for assay.

KAPA Mouse Genotyping Kit: DNA Amplification Protocol (general)

We do PCR reaction in 20 ul volume. By keeping reaction volume to ≤ 20 ul, we can use P20 Pipetman (instead of P200 Pipetman) to prepare reaction mixture.
10 ul aliquots are analyzed on agarose gel. The remaining 10 ul is for re-loading when required.
Most PCR machines accept 10 ul reaction volume if necessary (e.g., if genotyping kit becomes short supply).

1. Place 1 ul sample (prepared above) in PCR tube.

2. Prepare Master Mix (for 20 ul reaction volume): an example

	(*per reaction*)	
Water	5 ul	
10 uM Forward Primer	1 ul	
10 uM Reverse Primer	1 ul	
10 uM Internal Control Forward Primer	1 ul	
10 uM Internal Control Reverse Primer	1 ul	
2 x KAPA2G Fast Genotyping Mix	10 ul	- add last

   * Be aware that the 2x KAPA2G Fast Genotyping Mix forms gradient of the dyes during storage at -20°C. Mix and centrifuge before taking out aliquot.

3. Add & mix 19 ul aliquot of Master Mix to the sample in PCR tube.

4. Cycling parameters: an example

   95°C/3 min
   [95°C/15 sec, Ta (55-65°C)/15 sec, 72°C/10-30 sec per kb] x 35 cycles
   16°C

   Notes:
   - Annealing temperature (Ta): Tm ~ Tm+5°C (Tm is obtained by Primer3)
   - Ta given by ThermoFisher site (for Phire kit) appears to work well as the upper end of Ta for KAPA kit.
     https://www.thermofisher.com/ca/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html

- 10 sec/kb is recommended for most amplicons less than 1 kb.
- 35 cycles is “standard” cycle number. As long as good signal can be obtained, do not increase cycle number as it could yield false positives.

5. Analyze 10 ul aliquots on agarose gel.
   * Usually, 1.0 - 1.5% agarose gels suffice.

6. Take picture of the gel(s).

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Other Genotyping Kits

Multiple genotyping kits are available other than KAPA Mouse Genotyping Kit.
All, including KAPA kit, are based on a similar format.
They are designed to extract genomic DNA from ear or tail samples.
They provide "2 x concentrated ready-to-use reaction cocktail" for endpoint PCR.
They all work well for many genotyping assays.
Difference can be found in reliability and in price.
For most assays, KAPA Mouse Genotyping Kit works reliably - that's why it is our "staple" kit.
When it comes to price,however, KAPA Mouse Genotyping Kit is the most expensive.

Each kit contains different DNA polymerase.
Different DNA polymerases process individual assays differently.
And for that reason, there are a few assays which KAPA kit cannot handle very well.
Those assays may be dealt well with by other kits.
So we keep two other kits available and they are;

ThermoScientific Phire Tissue Direct PCR Master Mix, 1250 rxns
ThermoFisher/Invitrogen BioBar, cat# F-170L, $580.22 (November 2017)
* In one particular genotyping assay, we noticed that this kit often gave false-positives which appeared to have resulted from disproportionally amplified cross-contaminant. KAPA kit did not have such problem on same samples.
* We use this kit for one particular assay for which KAPA Mouse Genotyping Kit does not work well.

Quanta AccuStart II Mouse Genotyping Kit, 500 rxns
VWR, cat# CA89235-010, $506.46 (June 2017)
* We use this kit for one particular assay for which KAPA Mouse Genotyping Kit does not work well.

Manuals for those kits are available on line.

Salting-out Method for Genomic DNA Preparation

For "ordinary" genotyping assays that employ end-point PCR reactions with product size up to ~1 kb, use of commericial genotyping kit (such as KAPA Mouse Genotyping Kit) is highly recommended. The kits can save time which is often the most precious thing in the world.
However, in special situations, such as long-range PCR (LR-PCR) or sperm sample, the salting-out method described below gives far better and safer results.

The method below was originally designed for tail samples.
Therefore, for other types of samples, some modifications may be required.
For smaller tissue samples, such as ear notch, caution is required so as not to lose sample during ethanol precipitation/washing. Consider addition of glycogen as carrier at ethanol precipitation step.
For sperm samples, presence of DTT during proteinase-K digestion is necessary for recovery of genomic DNA. DTT helps dissociate protamines from genomic DNA.
For LR-PCR purpose, DO NOT VORTEX upon addition of ammonium acetate.

The old "proteinase-K digestion/phenol extraction/ethanol precipitation"-procedure is not described here because the salting-out method gives better result with less work.

Materials

Tail Prep Solution (50 mM Tris pH 7.5, 50 mM EDTA, 5% SDS)

To make up 500 ml;

125 ml	20% SDS
50 ml	0.5 M EDTA pH 8.0
25 ml	1 M Tris-Cl pH 7.5

Make up to 500 ml with dH_2O, with stirring.

ES Cell Lysis Buffer (100 mM NaCl, 20 mM Tris pH 7.5, 50 mM EDTA, 0.5% SDS)

To make 50 ml;

1 ml	5M NaCl
1 ml	1 M Tris-Cl pH 7.5
5 ml	0.5 M EDTA, pH 8.0
2.5 ml	10% SDS

Proteinase K (20 mg/ml in water: Store at -20°C)

Prepare stock solution in “10 mM Tris-Cl, pH 7.5, 20 mM $CaCl_2$, 50% glycerol” and store at -20°C.

* Some people dissolve proteinase K in pure water. Storage at -20°C in water, however, leads to precipitation of proteinase K.

10 M Ammonium Acetate (77 g in 100 ml water)

To make up 100 ml, dissolve 77.08 g of ammonium acetate crystals in 32.5 ml of water.

* The crystals do not get dissolved easily. Keep stirring in a beaker until it gets dissolved completely. Filter-sterilize into 50 ml tubes. Store working stock air-tight at room temperature. Store extra stocks at 4°C (although crystals may come out at low temperature).
* Keep crystals of ammonium acetate stock at 4°C in order to prevent loss of ammonia.
* Never warm-up the solution for the purpose of dissolving crystals, or ammonia would escape and pH would drop. Low pH would cause precipitation of EDTA.

20 mg/ml Glycogen (Roche, cat# 10901393001)

Procedure

1) Per tail, add 300 ul of tail prep solution & 15 ul of 20 mg/ml Proteinase K
    * For rather large chunk of tissues (spleen, kidney, muscle, fat, liver, etc.), add larger volume of tail prep solution & proteinase K proportionally. Do not try to reduce volumes of tail prep solution & proteinase-K too much.

   Per ear, add 100-300 ul of tail prep solution & 5-15 ul of 20 mg/ml Proteinase K
    * Tail & ear samples can be better processed with KAPA Mouse Genotyping Kit.

   Per 500 ul of cell lysate (in ES cell lysis buffer), add 5 ul of 20 mg/ml Proteinase K.

   Per 1 ul of sperm, add 300 ul of tail prep solution, 15 ul of 20 mg/ml Proteinase K, and DTT to 2 mM final.
    * See "Genomic DNA Extraction from Sperm" for more details.
    * Addition of DTT is very important. It helps dissociate protamine from DNA, thus aiding recovery of genomic DNA.

2) Digest at 55^{o}C overnight.
    * After the digestion, samples can be left at room temperature (not lower than room temperature) for many days, if not many weeks, without problem.

   If LR-PCR is planned, recover maximum amount of genomic DNA by "poking" tissue remains with tip (without sucking action) to disaggregate them completely.

3) Add 1/3 volume of 10 M ammonium acetate, mix well.
    * If 300 ul lysate, add 100 ul.
    * For majority of genotyping which employs end-point PCR with products shorter than ~ 1 kb, mix by vortexing for 15 seconds. The vortexing generates ~ 12 kb DNA fragments for easy handling.
    * If LR- PCR is planned, do not vortex. Mix by gentle inversions.

4) Spin at maximum speed for 1-2 minutes.

5) Transfer supernatant into labeled, clean tube
    * Use filtered tips to avoid cross-contamination.
    * For LR-PCR, re-spinning of the supernatant for removal of residual precipitates may be a good idea.

6) If very small piece of sample (such as ear notch or sperm) is being processed, add 1 ul of 20 mg/ml glycogen to the supernatant.
    * Glycogen works as carrier in ethanol precipitation.
    * Usually, no glycogen is necessary for tail samples.

7) Add 2.5 volumes of 95-100% ethanol.

8) Invert tube gently to precipitate DNA.
    * No problem even if precipitate is not visible.

9) Centrifuge for 1- 2 minute at maximum speed.
    * If no visibly detectable precipitate (e.g., ear samples), spin longer (e.g., 20 min).

10) Remove most of the supernatant.

11) Re-spin briefly and remove residual liquid.

12) Add to each tube ~1 ml of 70% ethanol, invert a couple of times to mix and then spin for $\geq$ 30 seconds, at maximum speed.

13) Remove most of the supernatant.

14) Re-spin briefly and remove residual liquid.

15) If long-range PCR is planned, add two more 70% ethanol washes (3-times total) in order to remove EDTA (from Tail Prep Solution).

16) Air dry.

17) Add appropriate volume of 1 x TE (e.g., 10 mM Tris-0.1 mM EDTA, pH 8), or just 5 ~ 10 mM Tris, pH 8. For long-range PCR, incubate at 4^{o}C overnight to aid rehydration.
    * For most ear samples, 200 uL of TE is a good volume. For LR-PCR, further dilution (e.g., $\geq$ 400 ul) may be preferred.